# ASPECTS OF L-GLUTAMATE DECARBOXYLASE AND GABA METABOLISM IN AVIAN BRAIN

by

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TO MY PARENTS

DECLARATION

I certify that I am the author of this thesis and that all the work included is my own unless otherwise indicated in the text.

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ABSTRACT

L-glutamate decarboxylase (GAD, EC.4.1.1.15), the enzyme responsible for the synthesis of the inhibitory neurotransmitter 4-aminobutyric acid (GABA) was partially purified from chick brain using a combination of column chromatographic (ion exchange, hydroxyapatite and gel filtration) and electrophoretic techniques. Preparative polyacrylamide gel electrophoresis resulted in enzyme inactivation and was, therefore, of limited use. Chick brain GAD proved to be unstable so attempts were made to develop an efficient affinity chromatography procedure (using the cofactor pyridoxal-5-phosphate (PLP) as the ligand) which would enable purification to homogeneity with a minimum number of steps. However, this technique depends on ready dissociation of PLP from the enzyme. Evidence is presented to suggest that the association between GAD and PLP is very tight and that dissociation does not occur easily.

Chick brain GAD has an $M_r$ of approximately 150 000 $\pm$ 15 000, calculated by gel filtration chromatography, and an apparent $K_m$ for its substrate glutamate of 3.7 $\pm$ 0.4 mM.

The localisation and regional variation of GABA metabolism in chick brain, from day 1 to day 14 post hatch, was investigated. The level of high affinity uptake of [$^3$H] GABA and the specific activities of GAD and 4-aminobutyrate:2-oxoglutarate transaminase (GABA-T, EC.2.6.1.19), the enzyme responsible for the degradation of GABA, were determined in homogenates of tissue taken from six different brain regions. This was complemented by histochemical localisation of GABA-T in thin (20 um) transverse sections of chick brain. It had been intended to localise GABAergic neurons by immunocytochemical techniques however, lack of a homogenous protein precluded conventional methods of raising antibodies in rabbits. Balb/c mice were injected with partially purified GAD and the spleen cells from an immune mouse fused with myeloma cells from the same strain of mouse. The hybrid cells produced were screened for anti-GAD activity. Two fusions were carried out in an attempt to raise monoclonal antibodies to chick brain GAD. From fusion 1, 3% of the wells seeded contained hybrids, and from fusion 2, 20% of the wells seeded contained hybrids. Preliminary screening experiments indicated that one of the hybrids formed from fusion 1 and several of those formed from fusion 2 may have secreted anti-GAD antibodies.

CONTENTS

## SECTION 1: INTRODUCTION

### 1.1 GABA - a major inhibitory neurotransmitter of the central nervous system

The amino acid 4-amino-butyric acid (GABA) is one of the most abundant amino acids in brain occurring at concentrations of between 1 and 10 μmol/g wet weight (Baxter 1976). GABA is distributed throughout the central nervous system (CNS), having been detected in almost every brain nucleus examined, and exhibits great topographical variations in concentration (Fahn & Côté 1968; Van der Heyden et al 1979). Although the presence of high concentrations of GABA in vertebrate CNS had been reported in 1950 (Roberts et al 1950; Undenfriend 1950; Roberts & Frankel 1950; Awapara et al 1950) and GABA had been identified as the active substrate which caused the inhibitory effects of crude brain extracts on the function of the crayfish stretch receptor system (Bazemore et al 1957), controversy as to the physiological role of GABA in the CNS continued for a number of years.

GABA is now accepted as a major inhibitory neurotransmitter in both vertebrate and invertebrate CNS. For reviews see Roberts (1974), Krnjević (1974), Roberts et al (1976), Krogsgaard-Larsen et al (1979). To summarise briefly there is both in vivo and in vitro evidence for a neurotransmitter role for GABA. Microiontophoretic application of GABA to neurons resulted in hyperpolarisation and changes in membrane conductance similar to those observed with the natural transmitter. GABA release has been demonstrated in vivo after stimulation of inhibitory neurons and in vitro using synaptosomes and tissue slices. GABA release has also been shown to have a calcium dependency. L-glutamate decarboxylase (GAD EC.4.1.1.15), the synthetic enzyme has been localised in the synaptosomal fraction of brain, has been demonstrated immunocytochemically at nerve terminals and has been shown to be concentrated in regions which have an inhibitory function and where, subsequently, GABA has been shown to act as a neurotransmitter. Finally a mechanism whereby GABA can be removed from the synaptic cleft has been demonstrated. This is a sodium and an energy dependent high affinity uptake system.

## 1.2 Difficulties associated with studying GABAergic systems in the CNS

Several characteristics of the GABA system complicate studies both of the role of GABA as a neurotransmitter and the regulation of GABA metabolism. GABA is ubiquitously distributed throughout the CNS and, unlike the monoamines and acetylcholine which are frequently restricted to specific brain nuclei, GABA or GAD activity can be measured in almost every brain nucleus examined (Fahn & Côté 1968; Tappaz et al 1976; Massari et al 1976; Van der Heydon et al 1979). Similarly receptor binding sites for GABA can be found throughout the CNS (Placheta & Karobath 1979; Olsen 1981).

An in vivo approach to studying the GABAergic system has advantages since tissue damage is kept to a minimum, the viability of the tissue is assured by an intact blood supply and the results of a particular experimental manipulation, e.g. the release of GABA evoked by stimulation of a certain structure or nerve tract, may be supposed to be a direct result of that physiological stimulation. However, in vivo studies are hampered by the complexity of the tissue which means that the experiments are technically difficult to carry out, for example, microiontophoretic application of a neurotransmitter substance to a group of cells without affecting any neighbouring cells. In addition localised tissue damage will occur during stimulation of nerve cells which may allow leakage.

Another complication which arises when studying amino acid neurotransmitters is that they are involved in the intermediary metabolism of the cell. In the case of GABA it has been estimated (using rat brain slices) that the GABA shunt (see Section 1.6) constitutes about 8% of the total carbon flux of the tricarboxylic acid cycle (Balazs et al 1970). Consequently there is a question as to whether GABA released in response to a physiological stimulus reflects a neurotransmitter role for the amino acid or a change in cellular metabolism caused by the stimulus.

The circuitry of the GABA system can also make it difficult to interpret or predict the specific functional result of a particular pharmacological manipulation. GABAergic neurons are often of the type which project over a short distance and are intrinsic to the nucleus, i.e. interneurons. In addition GABAergic neurons may synapse on each other (Vincent et al 1978) consequently augmentation of GABAergic transmission in one nucleus may indirectly cause inhibition of GABAergic activity in an adjacent area (see Section 1.4).

Compartmentalisation of GABA metabolism also poses problems when interpreting the results of *in vivo* experiments. GABA is synthesised primarily in GABAergic neurons, in particular at the axon terminals, (Fonnum & Walberg 1973) although there is somewhat controversial evidence that glia may also contain a low level of GAD activity (see Section 1.8). Removal of GABA from the synaptic cleft occurs by uptake into both nerve terminals and glial cells (Iversen & Kelly 1975). In addition, neuronal perikarya also have the capacity to take up GABA (East *et al* 1980) and all three of these compartments contain 4-aminobutyrate:2-oxoglutarate transaminase (GABA-T EC.2.6.1.19) (Robinson & Wells 1973; Kuriyama 1976; Barber & Saito 1976).

Consequently, the administration of drugs which affect GABA metabolism may affect all or only one of these compartments, not necessarily the nerve terminal compartment. As a result whole brain GABA concentrations may appear normal, or elevated, when the neurotransmitter GABA pool is markedly depleted or vice versa. Measurement of the whole brain GABA concentration is therefore of little value since changes in the GABA concentration of only one GABA pool or in only a few nuclei may be sufficient to markedly affect behavioural or physiological functions but would be undetected as a change in whole brain GABA.

As a consequence of the difficulties of studying the role of GABA in the CNS *in vivo* many investigations involving uptake and release of the amino acid have been made using tissue slice or synaptosomal preparations. Although experiments *in vitro* eliminate many of the technical problems associated with *in vivo* experiments and allow investigation of tissue from a specific region of the CNS, minimising tissue damage during preparation and maintaining tissue viability become major considerations. Results from *in vitro* experiments must, in addition, be interpreted with care with respect to the possibility that the mechanism observed *in vitro* may not be identical to the *in vivo* situation.

Several of the problems of interpretation associated with *in vivo* experiments also apply *in vitro* e.g. which tissue elements accumulate radiolabelled amino acids? This is still applicable when using synaptosomal preparations since even "pure" synaptosomal preparations are contaminated with gliasomes (Henn *et al* 1976). In addition, it is

not always altogether clear which pool radiolabelled amino acids enter, or are released from, during *in vitro* uptake and release experiments and whether this is the same pool as their endogenous counterparts.

## 1.3 GABA release

The demonstration of synaptic release of an electrophysiologically active substance in response to neural stimulation is of considerable importance for transmitter identification. Investigations involved in trying to demonstrate, and to determine the mechanism of GABA release in the CNS in response to a stimulus have encountered many of the problems both technical and of interpretation mentioned in the previous section.

Calcium-dependent release of GABA has been demonstrated *in vitro*, using tissue slices or synaptosomes which had been preloaded with [$^3$H] GABA (Srinvasan *et al* 1969; De Belleroche & Bradford 1972; Levy *et al* 1973; Redburn *et al* 1976; Cotman *et al* 1976), and *in vivo* (Obata & Takeda 1969; Roberts, P.J. 1974). Data obtained from these experiments were consistent with stimulus or depolarisation-induced release of neurotransmitter first proposed by Katz and Miledi (1967). In addition $Ca^{2+}$-independent release of GABA, which is in all other respects indistinguishable from the depolarisation-induced release process, has also been demonstrated (Srinivasan *et al* 1969; Obata & Takeda 1979; Fagg & Lane 1979).

Data from *in vitro* studies on the release of endogenous GABA from tissue slices (electrically-induced depolarisation) and synaptosomes (potassium-induced depolarisation) suggested that GABA released in a $Ca^{2+}$-dependent manner originated from a different pool from that released in a $Ca^{2+}$-independent manner (Valdes & Orrego 1978; Haycock *et al* 1978). It was suggested that $Ca^{2+}$- dependent release of GABA was from a bound (possibly vesicular) pool reflecting depolarisation-secretion coupling whereas $Ca^{2+}$-independent release might liberate GABA from a soluble pool by a membrane carrier system (facilitated by a depolarisation-induced influx of sodium into the terminals) and may reflect membrane transport processes. However, there is no evidence to date for vesicular storage of GABA and available evidence suggests a cytoplasmic rather than vesicular location for the neurotramsmitter (Mangan & Whittaker 1966; De Belleroche & Bradford 1973). In addition, results from subcellular fractionation studies suggested that GAD, the enzyme responsible for the synthesis of GABA, is a free cytoplasmic constituent of synaptosomes (Fonnum 1968).

Sandoval (1980(a)) put forward the proposal that depolarisation-induced release in $Ca^{2+}$-free medium may be due to an increased efflux of $Ca^{2+}$ from intracellular stores (primarily mitochondrial) elicited by the accumulation of sodium at nerve endings. This was based on several observations including that of a sodium-dependent efflux of calcium which has been demonstrated for mitochondria from excitable tissues (Carafoli & Crompton 1978), including brain, (Silbergeld 1977) but not for mitochondria from non-excitable tissues (Carafoli & Crompton 1978); that treatments which elicit $Ca^{2+}$ loss from mitochondria stimulate transmitter release from synaptosomes (Sandoval 1980(b)) and that increased sodium concentrations stimulated the release of recently accumulated [$^3$H] GABA (Sandoval 1980(a)).

In addition to depolarisation-induced release of GABA it has been suggested that there might be continuous coupled synthesis-secretion of GABA from the synaptosomes of inhibitory neurons which maintain tonic inhibition of the post synaptic neurons. Since release is dependent on GAD activity, inhibition of the enzyme activity would reduce this tonic inhibition and lead to convulsions (Tapia et al 1975). Additional observations that inhibition of GAD activity resulted in immediate convulsions, (Tapia et al 1967; Tapia et al 1969; Wood & Peesker 1973), that there is some correlation between the inhibition of GAD in nerve endings and the occurrence of convulsions caused by some drugs (Perez de la Mora et al 1973) and that in the presence of $Ca^{2+}$ GAD is bound to synaptosomal membranes (Fonnum 1968) supported this theory. Immunocytochemical localisation of GAD (at both the light and electron microscope level) has shown the enzyme associated with the presynaptic membrane although in addition there was some association with mitochondrial and synaptic vesicle membranes (McLaughlin et al 1975; Wood et al 1976). Further evidence that $Ca^{2+}$ ions promotes the association of GAD with membranes was obtained from experiments in which the effect of $Ca^{2+}$ on binding of GAD to liposomes was investigated (Covarrubias & Tapia 1978; 1980).

Although the mechanism by which GABA is released has not been clarified, depolarisation induces the release of the neurotransmitter in a manner which is at least partially dependent on exogenous $Ca^{2+}$ ions. In addition a second mechanism for GABA release has been postulated, that of coupled synthesis and secretion of the neurotransmitter.

## 1.4 Synaptic inhibition by GABA

GABAergic neurons can mediate both presynaptic and post synaptic inhibition. Post synaptic inhibition was the first to be demonstrated microiontophoretically (Curtis & Johnston 1974). GABA is released presynaptically and binds rapidly and reversibly to post synaptic membrane receptors (Enna et al 1975; Olsen et al 1979). This interaction regulates the opening and closing of membrane $Cl^-$ ion channels and results in increased membrane permeability to $Cl^-$ ions causing hyperpolarisation of the post synaptic neuron which reduces the likelihood of transmission of an excitatory impulse (Curtis & Johnston 1974; McBurney & Barker 1978; Curtis 1979).

In addition there is evidence that GABA can mediate presynaptic inhibition by causing a slow depolarisation of primary afferent fibres, also possibly associated with increased permeability to $Cl^-$ ions, (De Groat 1972; Fonnum 1978) by axo-axonal synapses. This causes a reduction in the amplitude of the action potentials in afferent terminals reducing their ability to release transmitter at the synapse.

Figure 1.1 is a schematic diagram showing four types of GABAergic synapse. Figure 1.1a illustrates presynaptic inhibition, impulses in primary afferent neurons (e.g. spinal and cranial nerves) are subject to presynaptic modulation by impulses from other afferents or from central structures before being transmitted to second order neurons. Other GABAergic neurons are involved in postsynaptic inhibition-GABAergic interneurons can form parts of local feedback inhibitory loops on principal neurons (e.g. in the cerebellar cortex) by collateral (Figure 1.1b) or recurrent (Figure 1.1c) inhibition. Both mechanisms allow the first excitatory impulses to fully activate a principal neuron whereas subsequent signals encounter a target cell which has been made less excitable by the preceding impulses thus preventing excessive excitation of neurons. Finally GABAergic neurons can directly transmit inhibitory influences from one central structure to another distant one (Figure 1.1d) for example, the cerebellar Purkinje cells which carry the total inhibitory output from the cerebellar cortex.

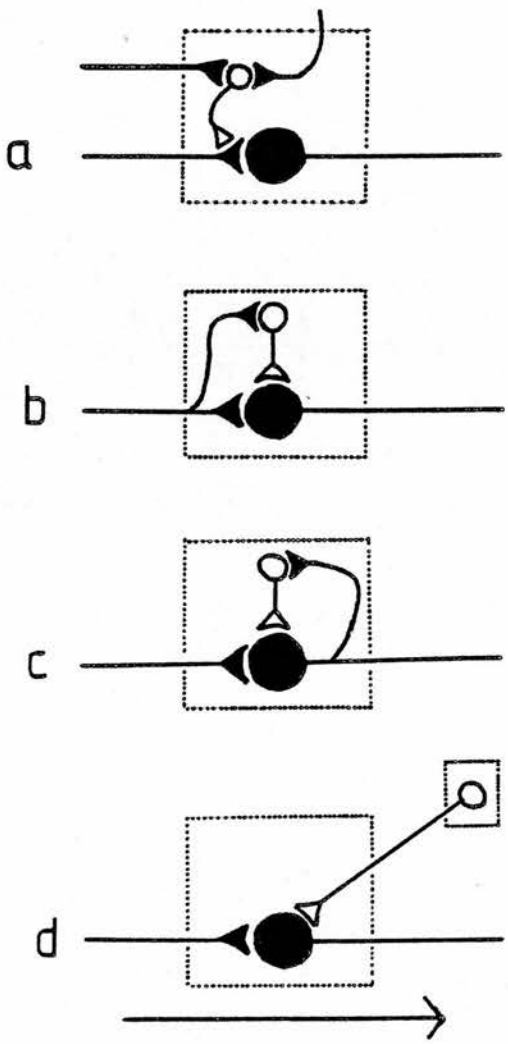



Figure 1.1 Schematic diagram of four types of GABAergic synapses. (Haefely et al 1978)

(a) Presynaptic inhibition
(b) Collateral inhibition
(c) Recurrent inhibition
(d) Post synaptic inhibition by projection neurons

See text, Section 1.4 for more details.

## 1.5 GABAergic systems and behaviour

GABA has become increasingly implicated in regulating the overall level of neuronal excitability throughout the CNS consequently, any interference with inhibitory synaptic transmission mediated by GABA has potentially a profound effect on the whole animal. Impairment of GABAergic transmission, whether at the level of GABA synthesis, release at the synapse or interaction with postsynaptic receptors, results in seizures (Tower 1976; Johnston 1978; Meldrum 1979).

Deficiencies in the GABAergic system have been implicated in a number of neurological disorders including Huntington's chorea , (McGeer & McGeer 1976; Enna 1977); Parkinson's disease, (Lloyd & Hornykiewicz 1973; Lloyd *et al* 1975; Lloyd *et al* 1977); epilepsy and schizophrenia (Tower 1976; Olsen *et al* 1980). Such deficiencies include a decrease in the number of GABA receptors, changes in receptor affinity for GABA, decreases in GABA level and changes in the level of GAD activity.

The importance of GAD in brain is due to the fact that it catalyses the rate limiting step in the synthesis of GABA. In addition, a number of studies have demonstrated that the GABA level in its active compartment is directly linked to presynaptic GAD activity (Iadarola & Gale 1980). Administration of drugs which inhibit GAD activity either specifically, for example, substrate analogues such as mercaptopropionic acid, or non specifically using for example carbonyl reagents such as hydrazides which react with pyridoxal-5-phosphate (PLP) result in the onset of convulsions (Meldrum 1979; Horton 1980). The immediacy of the effects of these drugs has suggested that GAD might be involved in a coupled synthesis-secretion mechanism (Tapia *et al* 1975).

The implication that the level of GAD activity in addition to the concentration of GABA in the neurotransmitter pool (rather than the GABA concentration in the whole brain) is an important factor in controlling neuronal excitability in the CNS indicates the importance of studies on the kinetics, catalytic mechanism and cellular regulation of GAD.

## 1.6 GABA metabolism

The predominant pathway for GABA biosynthesis in the CNS of both vertebrates and invertebrates is the irreversible decarboxylation of glutamate by GAD (Roberts et al 1958) with PLP as an essential cofactor (Roberts & Simonsen 1963).

The catabolism of GABA proceeds via two sequential reactions. The first step is the transamination with 2-oxoglutarate catalysed by GABA-T, which also requires PLP as a cofactor, to produce succinic semialdehyde (SSA) and glutamate. SSA is then oxidised by succinic semialdehyde dehydrogenase (SSADH EC.1.2.1.24) to yield succinate which can enter the TCA cycle. The GABA metabolic pathway is referred to as the GABA shunt since it provides an alternative metabolic pathway which bypasses the oxidation of 2-oxoglutarate to succinate (Roberts et al 1964; Baxter 1976; Cooper et al 1978). Figure 1.2 shows the GABA shunt and its relationship to cell intermediary metabolism.

## 1.7 Regulation of GAD activity

Although GAD is a key enzyme in the metabolism of GABA and has been implicated in the control of neuronal excitability (Tapia et al 1975) little is known about its regulation. It has been established that PLP is a necessary cofactor for the enzyme (Roberts & Simonsen 1963) however, the exact nature of the association between PLP and GAD is not known. It is thought, by analogy with other amino acid decarboxylases, that it forms a Schiff's base with a specific lysine residue on the enzyme polypeptide (Fasella 1967; Strausbach & Fischer 1970).

A number of studies, commonly using crude preparations such as cell cytosol, have been carried out to try to identify factors which influence GAD activity and might therefore form the basis of a control mechanism. It has been suggested that cofactor availability might, either directly or indirectly, constitute a means of regulating GAD activity (Baxter 1969; Tapia & Sandoval 1971; Bayon et al 1977(a) & (b); Miller et al 1978). Such a regulatory mechanism would depend greatly on the strength of the association between PLP and GAD, and this has been the subject of controversy.

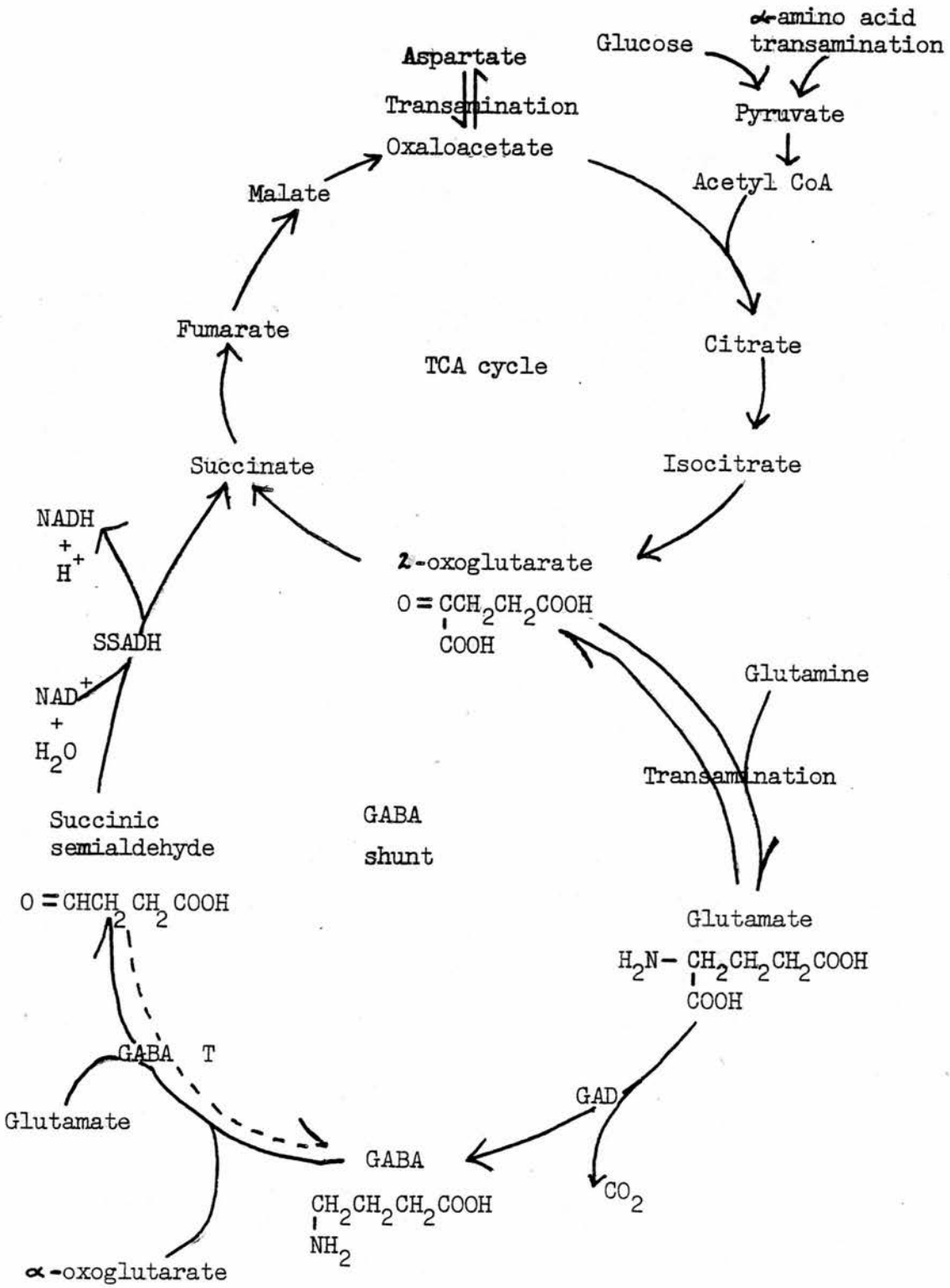



Figure 1.2 Schematic diagram showing the GABA shunt and its relationship to cell intermediary metabolism.

See text, Section 1.6 for further details.

In vivo evidence is contradictory, with evidence interpreted as suggesting that PLP may be loosely bound to the active site (Baxter 1969), so that cofactor availability would directly control enzyme activity, and also that PLP must be tightly bound to the enzyme since GAD activity was unaffected by changes in the PLP concentration in the brain (Gey & Georgi 1974). However, it is not surprising that such in vivo experiments should yield contradictory results since whole brain PLP levels do not necessarily reflect the levels in the neuronal GABA compartment. One of the major considerations when carrying out such experiments is that GABA exists in a number of compartments within the brain consequently it is uncertain whether drugs administered in vivo have depleted PLP in all the compartments containing GABA, in the neuronal GABA compartment or only in the non-neuronal compartments (see Section 1.2).

Data from in vitro studies using rat brain cytosol have been interpreted as suggesting that GAD may have two types of PLP binding sites, one which binds PLP very tightly, essentially irreversibly, and allow a basal level of GABA synthesis unaffected by cofactor concentration and another site which binds PLP loosely. In the latter case, occupancy of the PLP binding site would be directly dependent on the cofactor concentration and this would form the basis for a control mechanism since increased occupation would lead to increased enzyme activity. The data obtained did not predict whether the two sites were present on the same molecule, on different subunits of the same enzyme species or whether they were present on different enzyme species.

Miller et al (1978) suggested that the association between GAD and PLP was very tight in the absence of glutamate but that glutamate could promote dissociation of PLP from the active site. This was modified by Seligmann et al (1978) who proposed that, in the absence of ATP, available PLP reactivated enzyme which had been inactivated by glutamate-promoted dissociation, but that ATP blocked this reactivation. More recently Martin et al (1980) carried out similar experiments to those previously described (Miller et al 1978) replacing rat brain cytosol with partially purified hog brain GAD. The results were similar to those previously reported, however, inhibition of GAD by ATP was reported to be PLP independent in contrast with previous observations (Seligmann et al 1978). Factors which affect GAD activity and their implications for control of GABA synthesis are discussed in more detail in Section 7.

## 1.8 Non-neuronal GAD

For some time GAD in vertebrates was thought to exist exclusively in the CNS, although trace amounts of GABA had previously been detected in non-neural tissue (Roberts et al 1950). Subsequently low levels of GAD activity were detected in peripheral tissues such as kidney, heart, liver and blood vessels (Haber et al 1970(a); Whelan et al 1969; Kuriyama et al 1970; Wu 1977), in glia and gliomas (Haber et al 1970(b)) and in mitochondria from adult human and mouse brain (Haber et al 1970(c)). Several differences in the properties of the enzyme activity detected in these tissues, including stimulation rather than inhibition by aminooxyacetic acid (AOAA) and stimulation by anions such as chloride rather than inhibition, led to the concept of GAD II, a different glutamate decarboxylase from that found in neurons.

However, the concept of a different GAD based on differences in enzymological properties was refuted by reports showing that it was based on artefactual results (Miller & Martin 1973; Drummond & Phillips 1974; Kanazawa et al 1976; Wu et al 1978). In addition, Walsh and Clark (1976) could not repeat the results of Haber et al (1970(c)) and failed to detect any GAD activity in mitochondria. Drummond and Phillips (1974) reported that the GAD of mouse kidney appeared similar to neuronal GAD, based on enzymological properties, and suggested that the low levels of activity detected in peripheral tissues might be associated with nerve endings in that tissue.

However, there is evidence that peripheral tissues do contain low levels (5% or less of the activity of neuronal GAD, Wu 1980) of a different form of GAD since there is no immunological cross reactivity between mouse brain GAD and crude extracts from mouse peripheral tissues (Wu et al 1978; Wu 1980) or purified bovine heart GAD (Wu 1977; Wu 1980) and there are some slight differences in enzymological properties. Analysis of the products of $\alpha$-decarboxylation by the heart enzyme revealed, in addition to $[^{14}C]$ GABA, the presence of an unidentified $[^{14}C]$ labelled substance which was not produced by the neuronal enzyme.

There is evidence for a role for GABA in cerebrovascular function (Fujiwara et al 1975; Edvinsson & Krause 1979). Krause et al (1980) presented data indicating that GABA receptors and GAD and GABA-T activities were associated with cerebral though not peripheral blood vessels.

Subsequently low levels of GAD activity were detected in peripheral blood vessels (Hamel et al 1981). Further work revealed a number of differences between neuronal and cerebrovascular GAD activity suggesting that the latter may represent non-neuronal GAD. Consequently GABA formation in cerebral blood vessels may be manipulated independently from that in neuronal tissue (Hamel et al 1982).

## 1.9 Purification of GAD from CNS

The previous sections have shown the importance of GABA in the CNS and of GAD the enzyme responsible for GABA synthesis. Moreover, that there is a lack of definitive evidence for any particular mechanism by which GAD activity may be regulated. In addition, GAD has been generally accepted as a marker for GABAergic neurons (see Section 1.10) being regarded as more reliable than GABA since there is a possibility that the neurotransmitter may be metabolised or redistributed during tissue preparation. Consequently one of the aims of this project was to purify chick brain GAD to homogeneity in order to raise antibodies which could be labelled (e.g. FITC (See abbreviations)) and used for immunocytochemical localisation of GABAergic neurons in chick brain, in particular those involved in the visual system.

Neuronal GAD from several vertebrate species has been purified to homogeneity (Wu et al 1973; Maitre et al 1978; Blindermann et al 1978(a); Su et al 1979; Wu 1982). Table 1.1 shows some of the properties of rat and human GAD which are the most characterised to date. Initially attempts to purify this enzyme were unsuccessful due to its instability however, a combination of PLP and AET (See abbreviations) in all the buffers was found to improve the stability of the enzyme.

Enzyme instability resulting in loss of enzyme activity has proved to be a major problem in the purification of GAD since, for example, location of fractions containing GAD can only be achieved by assaying for enzyme activity. In addition, although GAD is present throughout the brain, mainly as a component of the synaptoplasm, it is not present in high concentrations. For example it has been estimated, by radioimmunoassay (Blindermann et al 1979) that the overall concentration of GAD in rat brain is $2.79 \pm 0.19$ pmol/mg protein (specific activity 164 nmol GABA produced/mg protein/hour) (Blindermann et al 1978(b)). Based on the molecular weight estimated for rat brain GAD (Maitre et al 1978) less than 0.04% of the total brain protein is GAD.

Table 1.1. Comparison of some properties of human and rat brain GAD (from Blindermann et al 1978(b))

| Property | Human enzyme | Rat enzyme |
|---|---|---|
| Molecular weight: | | |
| native | 140 000±15 000 | 140 000±15 000 |
| subunit | 67 000± 5 000 | 67 000± 5 000 |
| Disulphide bridged | None | None |
| N-terminal amino acid | α-alanine | α-alanine |
| $pH_i$ | 5.0±0.2 | 5.4±0.2 |
| pH optimum | 6.8±0.15 | 7.0±0.15 |
| $K_m$ glutamate | 1.28±0.07 mM | 1.10±0.07 mM |
| $K_m$ pyridoxal phosphate | 0.13±0.09 μM | 0.50±0.09 μM |

Since there is no particularly concentrated source of GAD large quantities of tissue are required to enable purification to homogeneity and to compensate for inactivation of the enzyme during purification. However, only a tiny percentage of the protein present in the initial tissue extract, e.g. supernatant, is GAD. Consequently, it is necessary either to remove much of the unwanted protein prior to purification or to process many small batches of tissue extract and pool the partially purified protein preparations before carrying the procedure through to homogeneity.

Successful purification of GAD has been achieved by two groups using slightly different protocols. In both cases an initial protein precipitation step was included. Ammonium sulphate fractionation was used several times in the purification of GAD from mouse, catfish and bovine brain (Wu et al 1973; Su et al 1979; Wu 1982) however, chick brain GAD was inactivated by this procedure (see Section 5). A similar observation was reported by Blindermann et al (1978(b)) who used a heat precipitation step (50°C for 5 minutes at pH 5.4) to reduce the protein content of the initial homogenates in the purification of human and rat brain GAD (Blindermann et al 1978(a); Maitre et al 1978). Although this step reduced the protein concentration by more than 80%, only 25% of the original GAD activity was retained.

Apart from the initial protein precipitation step the protocol used for the purification of rat and human brain GAD was similar to that used for chick brain GAD incorporating ion exchange, hydroxyapatite and gel filtration chromatography. The same methods were used in the purification of mouse, catfish and bovine brain GAD, although ammonium sulphate fractionation was included at several stages. In addition preparative gel electrophoresis was also used in the purification of catfish and bovine brain GAD (Su et al 1979; Wu 1982).

## 1.10 Affinity chromatography

Affinity chromatography is a technique which exploits a unique biospecificity of a protein for a ligand covalently bound to an insoluble support or matrix. An efficient affinity chromatography step incorporated at an early stage of the purification procedure could reduce the number of steps necessary to purify chick brain GAD, an important consideration since the enzyme is unstable.

PLP, an essential cofactor for GAD (Roberts & Simonsen 1963) was chosen as a suitable ligand since there is a strong association between GAD and PLP (Miller et al 1977). However, for maximal binding of an enzyme to a column where the ligand is a cofactor, it should be possible to aid the dissociation of the cofactor from the enzyme in such a manner that the enzyme is not damaged so that the solution which is applied to the column contains apoenzyme. Although PLP binds tightly to GAD, Miller et al (1978) put forward the proposal that in the presence of glutamate PLP dissociated (reversibly) from the enzyme and suggested that this could be a means of producing the apoenzyme.

Affinity chromatography using resin with a similar structure to that used for chick brain GAD (N'-(ω-aminohexyl)-pyridoxamine phosphate-Sepharose 4B) has been included in the purification of tyrosine amino-transferase (EC.2.6.1.5) (Miller et al 1972) and glutamate oxaloacetate transaminase (EC.2.6.1.1) (Collier & Kohlaw 1971).

Affinity chromatography has not been often used in the purification of GAD and in those instances where it was incorporated in the purification protocol it did not prove as successful as might have been anticipated. There have been three reports describing affinity chromatography of mammalian GAD. In one instance α-methyl glutamate, a competitive inhibitor of GAD, was used as the ligand (Yamaguchi & Matsumura 1977) and in the other PLP was covalently attached to a Sepharose 4B matrix through the aldehyde group (Possani et al 1977; Perez de la Mora et al 1981).

Possani et al (1977) achieved binding which ranged from 16% to 47% of the activity loaded (using a rat brain cytosolic fraction), however, the enzyme could apparently only be partially eluted from the resin. In the latter report the pellet obtained with 55% saturation of mouse brain cytosol with ammonium sulphate was resuspended and applied to the affinity column. Both the GAD which was eluted (1% of the activity in the homogenate) and that which remained bound to the resin was used to raise antibodies for immunocytochemistry.

However, SDS polyacrylamide gel electrophoresis revealed multiple bands both from the "soluble" GAD preparation and the bound GAD preparation similar to those obtained by Matsuda et al (1973), see also Section 5. Both these preparations, when injected into rabbits did cause the production of anti-GAD antibodies.

Following the preliminary report (Possani et al 1977) it was considered that large scale preparative affinity chromatography might be successful if apoenzyme, rather than a solution containing both holoenzyme and apoenzyme, was applied to column.

## 1.11 GAD as a marker for GABAergic neurons

There is good correlation between the regional distribution of high GABA levels and that of GAD (Kuriyama et al 1966; Fonnum and Walberg 1973; Fahn 1976). In addition, high levels of GAD activity have been shown immunocytochemically only in those terminals thought to be GABAergic (McLaughlin et al 1974; McLaughlin et al 1975(a); Saito et al 1974(a); Wood et al 1976). Consequently GAD has been generally accepted as a marker for GABAergic neurons. GAD as a neuronal marker has several advantages over the neurotransmitter, GABA, in that it can be demonstrated immunocytochemically and will not redistribute or be metabolised during tissue preparation.

Following the purification of mouse GAD (Wu et al 1973) and the production of antibodies to GAD (Matsuda et al 1973) a number of immunocytochemical studies of the distribution of the enzyme in the brains of several species have been reported (McLaughlin et al 1974; McLaughlin et al 1975(a); McLaughlin et al 1975(b); Wood et al 1976).

However, more recently human GAD which was judged to be pure by SDS polyacrylamide gel electrophoresis and isoelectric focussing proved to be able to catalyse a low level of decarboxylation of cysteine sulphinate (Blindermann 1978(a)). This suggests several possibilities; that GAD might be able to utilise cysteine sulphinate as a substrate under certain conditions, that the enzyme responsible for both glutamate decarboxylase and cysteine sulphinate decarboxylase activity might be the same entity or that GAD and cysteine sulphinate decarboxylase (EC.4.1.1.29) are so similar in structure that they copurified and could not be distinguished even by the fairly rigorous methods employed. Kinetic evidence did suggest that two different catalytic sites might be involved in the decarboxylation of glutamate and cysteine sulphinate (Blindermann et al 1978(a)).

The product of decarboxylation of cysteine sulphinate is hypotaurine, the immediate precursor of taurine which is a putative inhibitory transmitter (Cooper et al 1978). The same enzyme is thought to be responsible for the decarboxylation of cysteic acid to yield taurine

(Hope 1955; Cuion-Rain et al 1975). Consequently, this finding has important implications for the validity of identifying neurons as GABAergic using immunological techniques.

Oertel et al (1980) tried to separate GAD and cysteine sulphinate decarboxylase activities in order to raise antibodies to GAD alone for immunocytochemical studies. However, it did not prove possible to separate the two activities either biochemically or immunologically. Wu (1982) using the protocol devised for the purification of mouse brain GAD with the addition of a final preparative gel electrophoresis step, separated two enzyme entities from bovine brain. One was responsible for the decarboxylation of cysteine sulphinate and cysteic acid with $K_m$ values for the two substrates of 0.22 mM and 0.18 mM respectively, the other decarboxylated glutamate, $K_m$ = 1.6 mM, but could also decarboxylate cysteine sulphinate and cysteic acid with $K_m$ values of 5.2 mM and 5.4 mM respectively. It was found that although the two enzymes copurified through several procedures they could be separated by hydroxyapatite chromatography and were immunologically distinct.

It may be concluded therefore that GABA and taurine are the products of two different enzymes. However, GAD, at least under certain in vitro conditions, does have the capability of decarboxylating cysteine sulphinate and cysteic acid. This finding would appear to reconfirm the validity of immunocytochemical localisation of GABAergic neurons. Unequivocal evidence for the identity or non identity of the two enzyme entities, and consequently different types of neurons, could be established using monoclonal antibodies. The specificity of monoclonal antibodies for one single determinant enables a distinction between two entities which may not be possible using conventional heterogeneous antisera which are polyclonal in nature (see Section 1).

## 1.12 Monoclonal antibodies

Until relatively recently the only method of obtaining antibodies to a given substance was to immunise an animal (e.g. rabbit, goat, sheep, horse) with a homogeneous preparation of the substance. When the animal had reached a hyperimmune state a blood sample was removed since the antibodies of interest would constitute a high percentage of the immunoglobulin content of the serum. However, the antibody response to a given substance is highly heterogeneous. When an animal is injected

with an immunogen it responds by producing diverse antibodies against different antigen molecules of the immunogen and against different determinants on a single antigen. In addition antibodies against other unrelated antigens with which the animal has come into contact will be present in the serum. It is virtually impossible to separate all the various antibodies so conventional antisera contain mixtures of antibodies which vary with different animals.

The development of the monoclonal antibody technique (Kohler & Milstein 1975; Kohler & Milstein 1976; Galfré et al 1977) has removed the necessity of using a pure antigen to stimulate the production of antibodies and has resulted in the ability to produce monospecific antibodies to a specific determinant in a reproducible manner. The technique involves fusing myeloma cells (myelomas are tumours of antibody secreting cells that arise spontaneously in animals but can also be induced) with naturally occurring lymphocytes from the same species which proliferate in the spleen and other lymphoid organs as a result of antigenic stimulation. Lymphocytes have a very short life span and cannot be cultured in vitro, however, codominant expression of immortality (conferred by the malignant phenotype of the myeloma parent) and specific antibody production (a property of the lymphocytes) is achieved in the hybridoma cells which are the result of the fusion.

After fusion, hybridoma cells are selected for by growth in HAT (hypoxanthine, aminopterin, thymidine) selective medium (Littlefield 1964). The presence of aminopterin in the medium blocks de novo synthesis of nucleotides (see Figure 1.3). Aminopterin inhibits dihydrofolate reductase preventing the formation of tetrahydrofolate and therefore the transfer of methyl groups in the de novo synthesis of nucleotides. To survive cells are, therefore, dependent on the second "salvage" pathway of nucleotide synthesis which relies on active hypoxanthine (guanine) phosphoribosyl transferase (H(G)PRTase EC2.4.2.8) and thymidine kinase (TK EC.2.7.1.21) for incorporation of the hypoxanthine and thymidine present in the medium. The parent tumour cell line is selected for deficiency in H(G)PRT ase, consequently, only the hybridoma cells (which have inherited an active salvage pathway from the parental lymphocytes) survive this treatment.

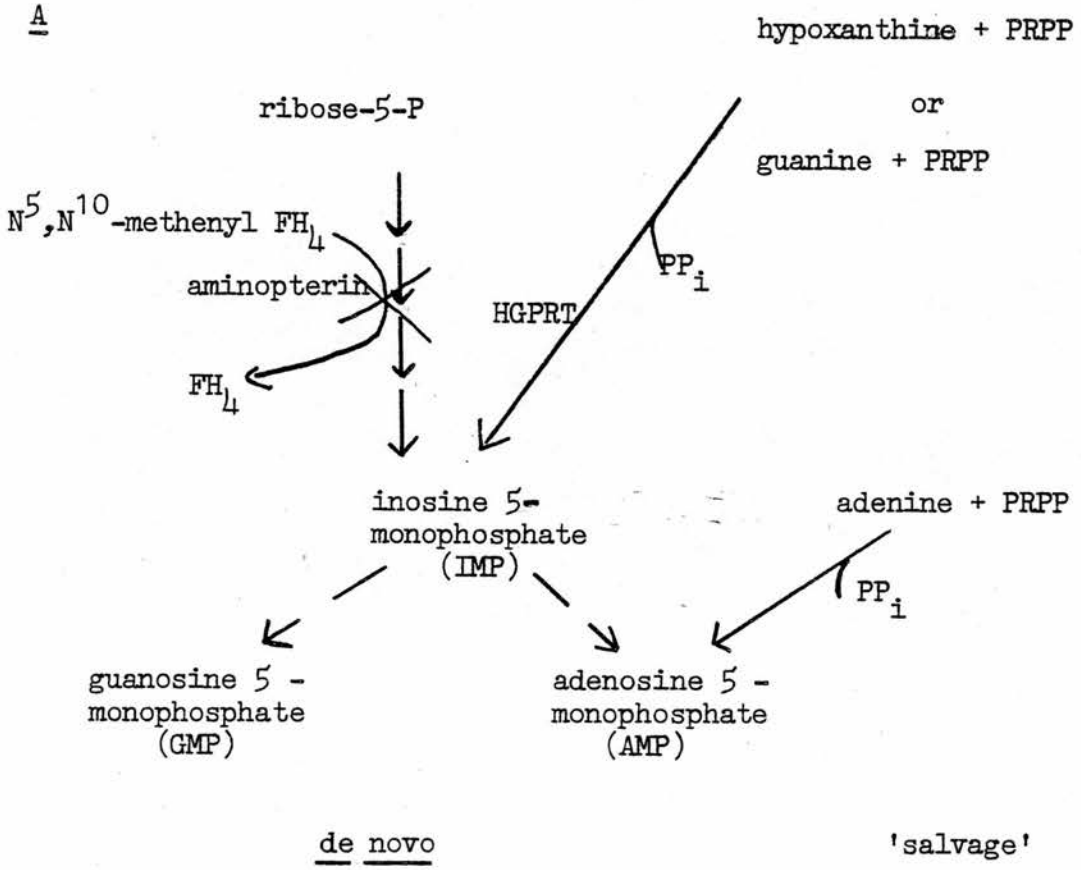
Figure 1.3 Schematic diagram showing the effects of growth in HAT selective medium on cell nucleotide metabolism.

A Purine nucleotide biosynthesis

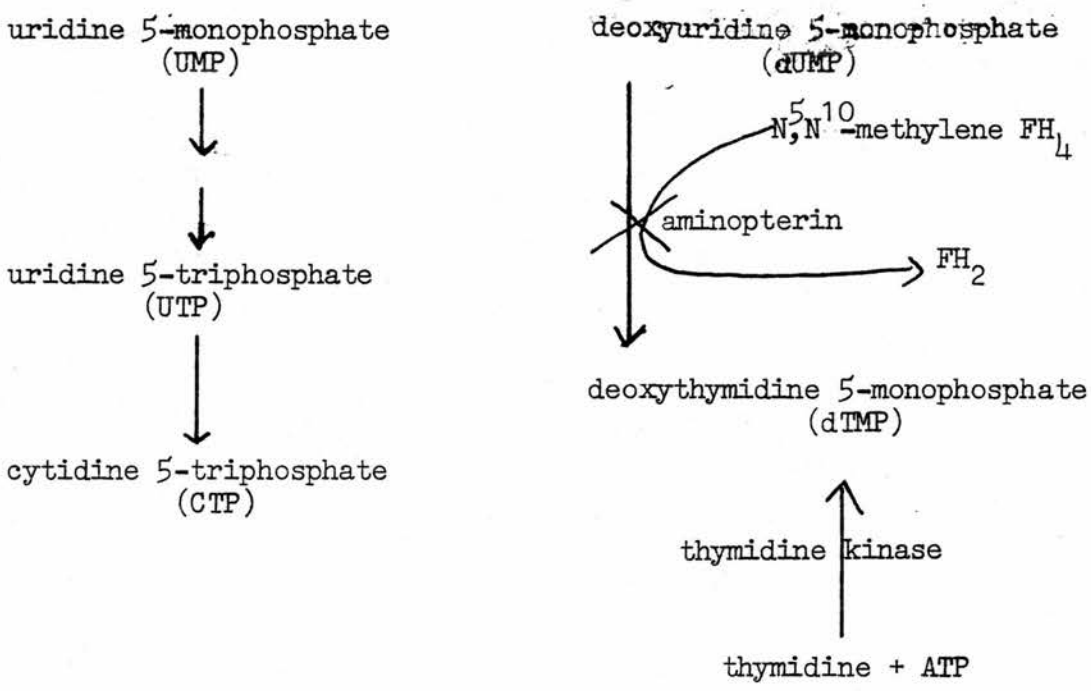
B Pyrimidine nucleotide biosynthesis

See text, Section 1.12

A


hypoxanthine + PRPP
or
guanine + PRPP
ribose-5-P
$N^5, N^{10}$-methenyl $FH_4$
aminopterin
$FH_4$
HGPRT
$PP_i$
inosine 5-monophosphate (IMP)
adenine + PRPP
$PP_i$
guanosine 5-monophosphate (GMP)
adenosine 5-monophosphate (AMP)
de novo
'salvage'

B


uridine 5-monophosphate (UMP)
uridine 5-triphosphate (UTP)
cytidine 5-triphosphate (CTP)
deoxyuridine 5-monophosphate (dUMP)
$N^5, N^{10}$-methylene $FH_4$
aminopterin
$FH_2$
deoxythymidine 5-monophosphate (dTMP)
thymidine kinase
thymidine + ATP

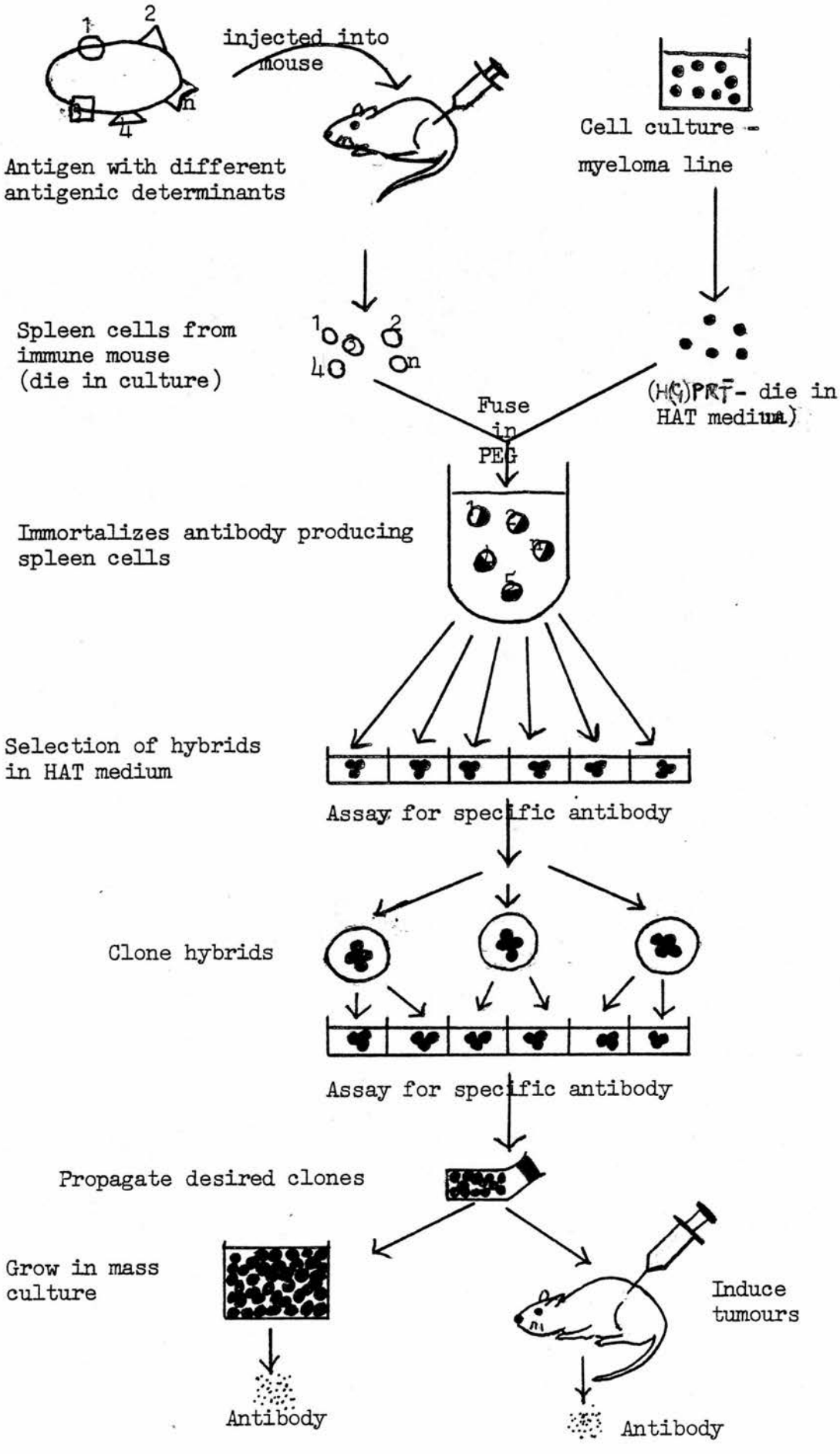
The parental tumour cell line is also selected for the inability to secrete immunoglobulin (or preferably the inability to synthesise immunoglobulin) since cell lines resulting from the fusion of two immunoglobulin producing cells secrete new immunoglobulins as a result of heterologous association of both parental heavy and light chains (Kohler & Milstein 1975). This may be avoided by using a non-secreting variant such as NS-1 (a Balb/c mouse cell line) which although it synthesises light chain molecules does not secrete them. However, after fusion it is possible that the myeloma light chain may be secreted as part of a new hybrid immunoglobulin molecule (Kohler et al 1976). The steps involved in the production of monoclonal antibodies are shown in Figure 1.4.

There are two major advantages of this technique. Firstly, it enables the production of monospecific antibodies from non-purified antigens. This is particularly useful if instability or low tissue concentration of the antigen in question prevents purification to homogeneity so that antibodies cannot be raised using conventional techniques. Secondly, the monoclonal antibody produced by an isolated clone is a well defined substance active against one single determinant, rather than a heterogenous mixture of antibodies which changes with each immunised animal and even with each bleeding of the same animal. Once stable hybridomas are established, an unlimited supply of antibodies of exactly the same chemical structure can be produced from permanent tissue cultures. Alternatively large amounts of the same monoclonal antibodies can be produced from tumours grown in animals.

The first permanent cell lines secreting a predefined antibody were against sheep red blood cells (Kohler & Milstein 1975), the hapten 2,4,5-trinitrophenyl (TNP) (Kohler & Milstein 1976), histocompatability and other cell surface antigens (Galfré et al 1977; Williams et al 1977). Subsequently many other cell lines which secrete monoclonal antibodies have been produced and it would appear that the procedure is general, i.e. that any antibody which an animal can produce can also be prepared as a monoclonal antibody through hybrid cell lines. The degree of difficulty in obtaining a specific hybrid myeloma is probably correlated with the immune response of the immunised animal so that if the response is very weak it will be correspondingly difficult to isolate a clone producing the desired antibody from amongst the many hybrids secreting unwanted immunoglobulins.

Figure 1.4 Scheme showing a standard procedure used for the production of monoclonal antibodies.

See text, Section 1.12 for further details; Section 8.1.5 contains experimental details.


injected into mouse
Antigen with different antigenic determinants
Cell culture - myeloma line
Spleen cells from immune mouse (die in culture)
(HGPRT- die in HAT medium)
Fuse in PEG
Immortalizes antibody producing spleen cells
Selection of hybrids in HAT medium
Assay for specific antibody
Clone hybrids
Assay for specific antibody
Propagate desired clones
Grow in mass culture
Induce tumours
Antibody
Antibody

## 1.13 Avian visual pathways

The previous sections have outlined several aspects of GABAergic systems in general and have tried to illustrate the importance of the amino acid in the CNS. It was of interest to apply some of the techniques mentioned earlier in this section to investigate biochemically and immunohistochemically the extent to which GABA was involved in a specific pathway within the CNS, namely the visual system.

The avian visual system was chosen as a model to investigate the role of GABA in the visual processes since it presents several advantages as a neurobiological model. Despite the relatively small size of the avian brain, the visual system is highly developed with prominent and therefore easily accessible optic lobes. The retinal input is almost completely crossed at the optic chiasma allowing experiments to be performed on one side of the brain whilst using the other as a control. In addition, the avian visual system has been extensively studied morphologically, physiologically, biochemically and behaviourally.

In the avian visual system two pathways projecting from the retina have been described, the tectofugal pathway and the thalamofugal pathway (Karten 1969; Karten & Hodos 1970; Karten et al 1973). The tectofugal pathway extends from the retina to the superficial layers of the contralateral optic tectum and thence to the nucleus rotundus in the midbrain and finally to the ectostriatum in the forebrain. The thalamofugal pathway projects from the retina to the contralateral dorsolateral thalamus and then to the Wulst in the forebrain (see Figure 1.5).

The optic tectum, which is a highly organised, laminated structure, is quantitatively the most important first relay in the avian visual pathway connecting the retina with the thalamus, pretectum and lower brain stem. This tectal relay system receives additional inputs from the visual Wulst (hyperstriatum) nucleus spiriformis lateralis and the contralateral tectum. There is a loop of reciprocal connections between the tectum and the nucleus isthmi pars parvocellularis. The tectum also projects to nucleus isthmo opticus which in turn sends efferent fibres to the retina (Cuénod & Streit 1979). In addition many intrinsic neurons and interneurons are present within the various tectal layers (Karten 1969; Meier et al 1974; Hunt & Webster 1975; Hunt & Kunzle 1976(a); 1976(b); Streit & Reubi 1977) (see Figure 1.6).

Abbreviations

| | |
|---|---|
| Ch. Op. | Chiasma opticum |
| E | Ectostriatum |
| Ep | Zona periectostriatalis |
| GLv | N. geniculatus lateralis pars ventralis |
| HA | Hyperstriatum accessorium |
| HD | Hyperstriatum dorsale |
| HV | Hyperstriatum ventrale |
| IHA | N-intercalatus hyperstriatum accessorium, int = lamina internus, ext = lamina externus |
| N | Neostriatum |
| OPT | N. optic principalis thalami dorsalis |
| PA | Paleostriatum augmentatum |
| PP | Paleostriatum primitivum |
| Rt | N. rotundus thalami |
| SGC | Stratum griseum centrale |
| SGF | Stratum griseum fibrosum centrale |
| TeO | Tectus opticum |
| Tr.Op | Tractus opticus |
| Tr.Rt-E | Tractus rotundus ectostriatalis |

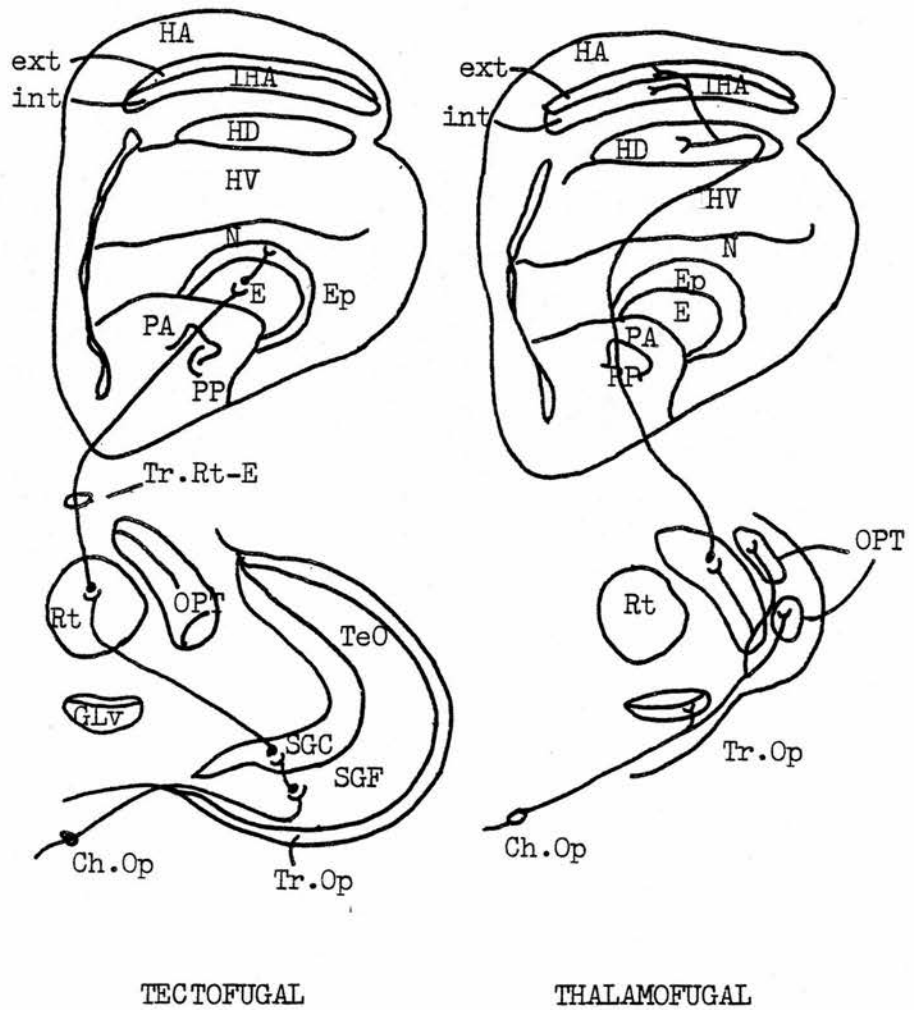
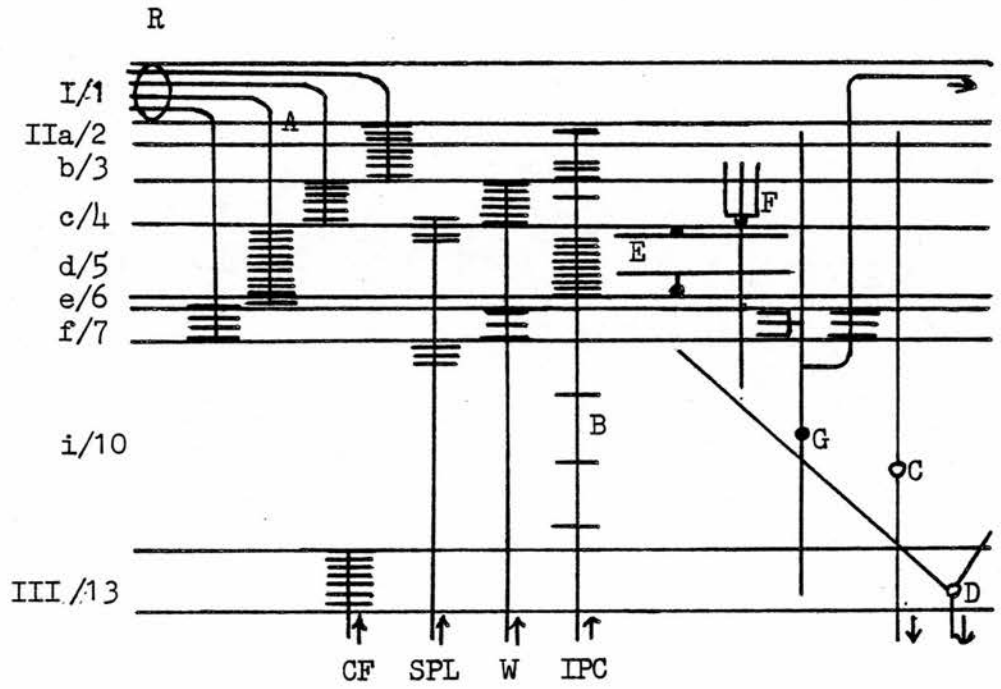



Figure 1.5 Schematic summary of two major recognised ascending visual projection pathways to the telencephalon, the tectofugal pathway and the thalamofugal pathway. (from Karten et al 1973) See text, Section 1.13 for further details.

Figure 1.6. Schematic representation of the input-output relationship in avian optic tectum (Cuénod and Streit 1979).

Layer nomenclature according to Cowan et al 1961 (roman numerals with subdivision of layer II) and Ramón y Cajal (1891) (layers 1-13).

A, optic afferent fibres; B, Ipc afferent fibre; C, neuron projecting to Ipc; D, large efferent neuron; E, horizontal intrinsic neuron; F, radial intrinsic neuron; G, neuron with terminals in superficial tectum and pretectum.




| | AFFERENT | INTRINSIC | EFFERENT |
|---|---|---|---|
| TELENCEPHALON | Wulst(W) | | - |
| DIENCEPHALON | Retina(R) | | Rotundus<br>DLL<br>GL<br>Pretectum |
| MESENCEPHALON | Ipc<br>SpL<br>Contra-tectum(CF) | | Ipc<br>ION |

DLL, N.dorsolateralis anterior thalami, pars lateralis; GL, N.geniculatus; ION, N.isthmo-opticus; Ipc, N.isthmi, pars parvocellularis; SpL, N. spiriformis lateralis.

The optic tectum has been intensively studied biochemically to elucidate the possible rotes of various neurotransmitters. There is biochemical and electrophysiological evidence to suggest that the inhibitory neurotransmitter GABA plays a prominent role in avian optic tectum. Synaptosomes prepared from optic tectum had a high affinity uptake mechanism for GABA (Henke et al 1976) whilst GAD has been shown to be present in optic tectum, particularly within the superficial layers (Henke & Fonnum 1976). Microiontophoretic application of GABA resulted in the inhibition of a large number of neurons, again particularly within superficial tectal regions, and that intertectal inhibition could be abolished by the application of the GABA antagonist bicuculline (Barth & Felix 1974). Light microscopic autoradiography after injection of [$^3$H] GABA into optic tectum revealed that four types of neurons had been specifically labelled, two of them intrinsic and two extrinsic. One type of GABAergic intrinsic neurons had their cell bodies in sublayer IIc (4) with short processes going radially into layer II whilst the others ran horizontally within layer IId (5) and IIc (4) and had long dendrites and short axons (see Figure 1.6). The extrinsic GABAergic neurons projected from optic tectum into the pretectum and ventral thalamus and also from the ventral part of the optic tectum to the anterior portion of nucleus isthmi pars parvocellularis. This connection between nucleus isthmi pars parvocellularis and optic tectum is reciprocal (Hunt & Kunzle 1976(b)).

Although there is much information available regarding the optic tectum, little is known about the role of neurotransmitters in other structures of the avian visual system which have been shown to play an important role in the visual processes (Hodos & Karten 1966; Hodos 1969; Hodos & Karten 1970; Karten et al 1973). In addition, much of the evidence regarding the identification of specific neurons within and projecting from the optic tectum as GABAergic has been obtained by light microscopic autoradiography following injection of [$^3$H] GABA (Henke & Fonnum 1976).

However, there are a number of factors which may reduce the specificity of the technique and complicate the interpretation of the data. These include difficulties in siting the injection correctly, diffusion of the radiolabelled amino acid from the site of the injection and high and/or low affinity uptake into non-neuronal elements such as glia and

perikarya of non-GABAergic neurons. Immunohistochemical techniques using antibodies to neuronal GAD, particularly a specific monoclonal antibody, provide a means of determining whether a particular neuron is GABAergic. One of the aims of the project was to use such immunohistochemical techniques to trace GABAergic neurons within the avian visual system in conjunction with biochemical techniques to determine the levels of GABA metabolism in those structures involved in the visual process.

## SECTION 2: MATERIALS

One day old shaver chicks were obtained from the Poultry Research Centre, Roslin, Midlothian. Wistar rats and Balb/c mice were obtained from animal house stocks.

L-[U-$^{14}$C] glutamate (1.85-2.2 GBq/mmol) and [2,3-$^{3}$H] GABA (1.85-2.6 TBq/mmol) were purchased from Amersham International, plc., Buckinghamshire.

All constituents of tissue culture media were purchased from Gibco, Paisley, Scotland.

Sheep immunoglobulin G against whole mouse serum was obtained from the Scottish Antibody Production Unit, Law Hospital, Carluke.

Cholera toxin and phosphorylase b were a gift from Dr W Ward, Department of Biochemistry, University of Edinburgh.

Toluene, Triton X100 and PPO were scintillation grade and obtained from Koch Light Laboratories Ltd.

All other chemicals, buffer constituents and substrates were obtained from BDH Chemicals Ltd. and were AnalaR grade, or from Sigma, London, unless otherwise indicated in the text.

Tissue Tek II OCT embedding medium was obtained from Lab-Tek Products, Division Miles Laboratories Inc. and DEPEX mounting medium from Gurr, Searle Diagnostic, High Wycombe, Bucks.

## SECTION 3: GENERAL METHODS

### 3.1 Column chromatography

All the buffers which were used to equilibrate or elute chromatographic columns used in the purification of GAD contained 0.1 mM PLP and 1 mM AET.

Standard buffer consists of 50 mM potassium phosphate containing 0.1 mM PLP and 1 mM AET, pH 7.4.

#### 3.1.1 Gel filtration chromatography

All gel filtration chromatography was carried out at 4°C. The gels used, Sephadex G200 and Sephadex G25, were swollen and the gel bed packed in the column in the manner recommended by the manufacturers (see "Sephadex, Gel Filtration in Theory and Practice", Pharmacia Fine Chemicals).

##### 3.1.1.1 Gel filtration using Sephadex G200

Calibration of the column

A column of Sephadex G200 (1.8 x 95 cm) was equilibrated in standard buffer. The column was calibrated with a selection of proteins as shown in Table 3.1. Figure 3.1 shows the elution profile obtained when a solution of the proteins in blue dextran and fluorescein was applied to the column and eluted with standard buffer at 10 ml/hour. Protein was monitored by measuring absorbance at 280 nm. In this way the partition coefficient, $K_D$, could be related to the apparent $M_r$ (see Figure 3.2).

$K_D$ is given by:

$$K_D = \frac{V_e - V_o}{V_s}$$

where $V_e$ = the elution volume for a particular protein

$V_o$ = the elution volume for molecules completely excluded from the gel

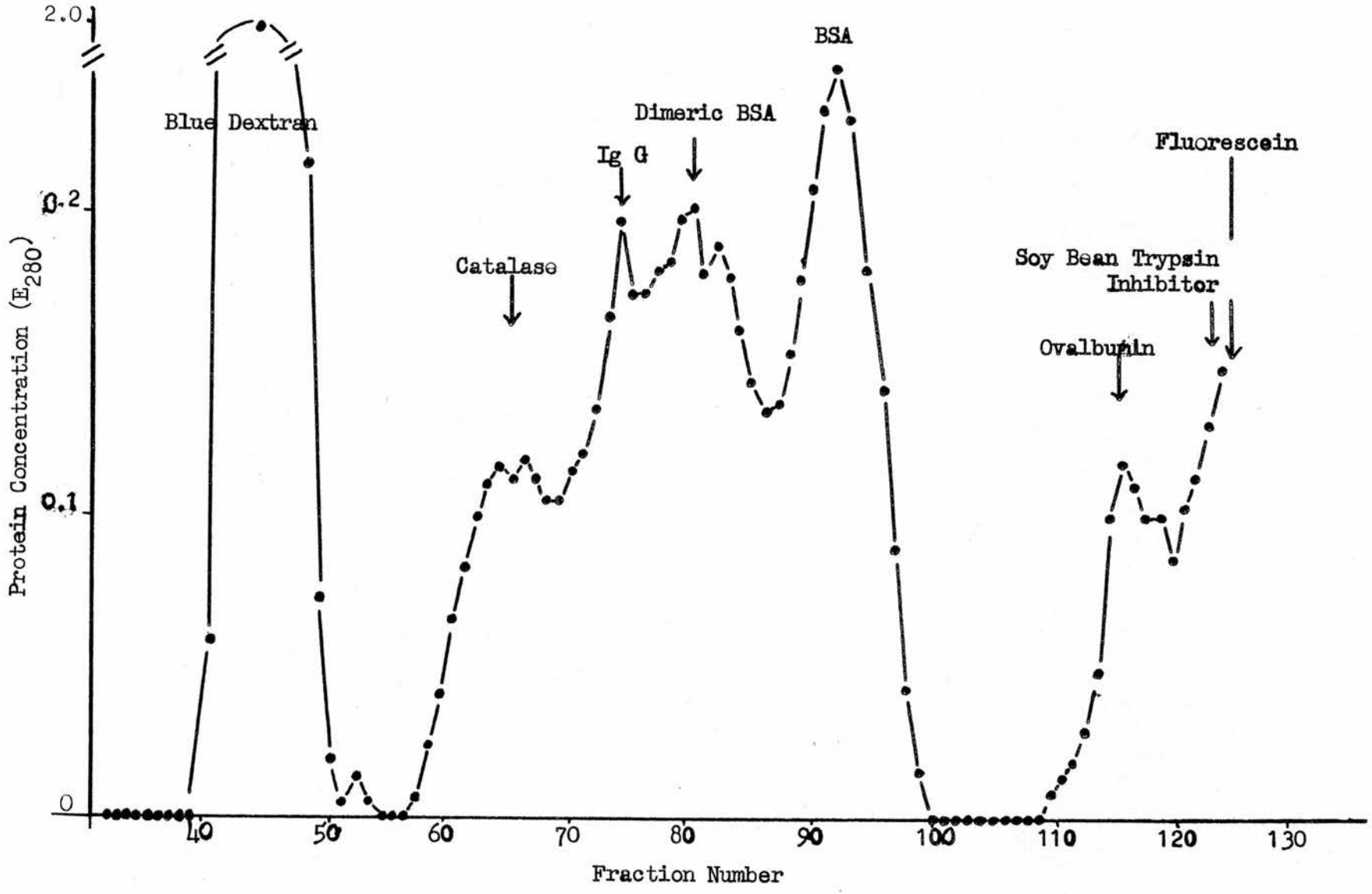
$V_s$ = the elution volume for molecules which readily diffuse into the gel beads.

A semilogarithmic plot of $K_D$ versus log $M_r$ (Figure 3.2) shows a linear relationship over the range of $M_r$ used. The line of best fit was calculated using the nonparametric method of Nimmo and Atkins (1979). From this calibration the Sephadex G200 column could be used to fractionate proteins of apparent $M_r$ between 20 000 and 300 000.

Table 3.1. $M_r$ standards for gel filtration chromatography

| Protein | Source | $M_r$ | Reference |
|---|---|---|---|
| Trypsin inhibitor | Soy Bean | $21.5 \times 10^3$ | Wu and Sheraga (1962) |
| Ovalbumin | Egg | $43 \times 10^3$ | Weber and Osborn (1969) |
| Serum albumin (BSA) | Bovine | $68 \times 10^3$ | Weber and Osborn (1969) |
| Dimeric serum albumin | Bovine | $136 \times 10^3$ | Weber and Osborn (1975) |
| Immunoglobulin G (IgG) | Bovine | $147 \times 10^3$ | Weber and Osborn (1975) |
| Catalase | Bovine liver | $232 \times 10^3$ | Darnall and Klotz (1975) |

Figure 3.1 Elution profile obtained when the proteins listed in Table 3.1 were used to calibrate a column of Sephadex G200 (1.8 x 95 cm). See section 3.1.1.1. Protein was monitored by measuring absorbance at 280 nm. Fraction size = 1.2 ml.


2.0
0.2
0.1
0
Protein Concentration ($E_{280}$)
Blue Dextran
Catalase
Ig G
Dimeric BSA
BSA
Soy Bean Trypsin Inhibitor
Ovalbumin
Fluorescein
40
50
60
70
80
90
100
110
120
130
Fraction Number

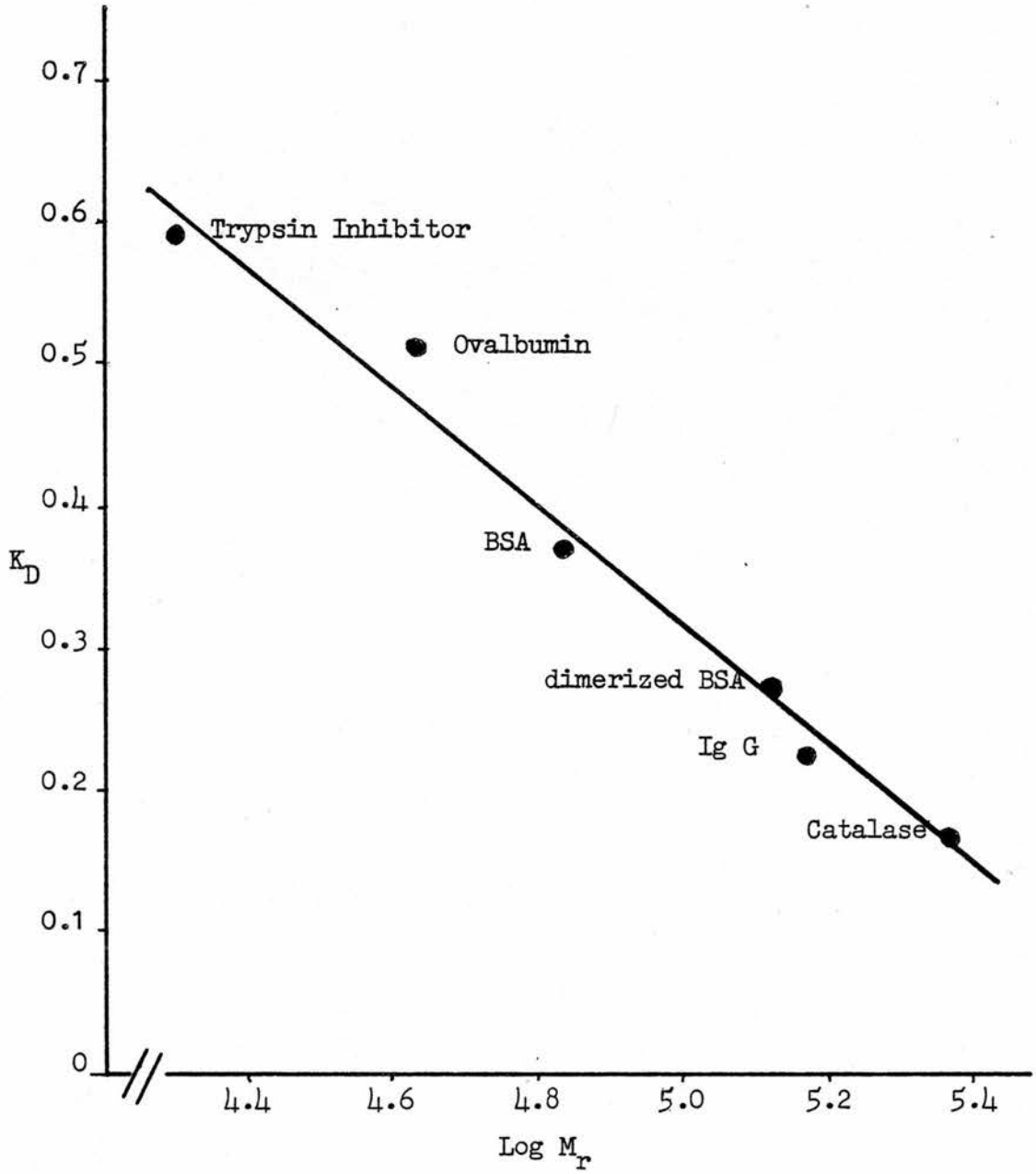



Figure 3.2 A graph of $K_D$ versus log $M_r$ for the proteins (listed in Table 3.1) used to calibrate the Sephadex G200 column. See section 3.1.1.1. The best straight line was calculated using the nonparametric method of Nimmo and Atkins (1979)

This column was routinely used during the purification of GAD (see Section 5). In order to determine where GAD activity was eluted in relation to the void volume, $V_o$, and the bed volume, $V_s$, and hence assign an apparent $M_r$ to the protein, on several occasions samples containing GAD activity were mixed with blue dextran (0.2% w/v), and fluorescein before application to the column. Elution with standard buffer was at 10 ml/hour.

3.1.1.2 Desalting by gel filtration using Sephadex G25

Gel filtration using Sephadex G25 was selected as a fast and efficient method of removing salts and other low molecular weight compounds from protein solutions containing GAD activity. Since separation of compounds of high and low molecular weight is very good, large sample volumes of up to 30% of the total bed volume can be used (Flodin 1961). This is particularly useful when desalting large volumes of supernatant prior to the purification of GAD.

Approximately 150 ml of a supernatant, prepared as described in Section 5.1, was loaded onto a Sephadex G25 column (5.4 x 27 cm) which had been previously equilibrated in standard buffer. Protein was eluted with standard buffer. Usually two such columns were used to prepare sufficient material to load onto the DEAE cellulose column (see Section 5.1). Protein was monitored by measuring absorbance at 280 nm and the protein peak applied to the DEAE cellulose column.

A similar procedure was employed for desalting the pooled peak fractions obtained from each DEAE cellulose column prior to chromatography using hydroxyapatite (see Section 5.1). Standard buffer was replaced with 5 mM potassium phosphate containing 0.1 mM PLP and 1 mM AET, pH 7.4.

Phosphate assays (see Section 3.6.3) which were performed on the eluates from the Sephadex G25 columns, prior to chromatography on hydroxyapatite, confirmed that the phosphate concentration was 5 mM.

It was confirmed that high and low molecular weight compounds were completely separated under the conditions described above by applying 150 ml of a mixture of blue dextran, 0.2% (w/v), and fluorescein on to a Sephadex G25 column, which was identical to those used for desalting, and eluting using similar conditions.

3.1.2 Ion exchange chromatography

3.1.2.1 DEAE cellulose

All ion exchange chromatography using DEAE cellulose was carried out at 4°C using Whatman DE 52 DEAE cellulose. The DEAE cellulose was degassed, equilibrated with standard buffer and packed in the column using the methods recommended by the manufacturers in their information leaflet 1 L 2. Experiments using DEAE cellulose are described in Section 5.1.

3.1.2.2 Dowex AG 1 x 2

Conversion of Dowex AG 1 x 2 (100-200 mesh) to the acetate form

This is a two step process, firstly ion exchange as the chloride is replaced by the hydroxide ion, followed by neutralisation of the hydroxide using acetic acid.

The hydrated resin, generally about 250 ml of a thick slurry, was poured into a wide column and washed with 20 volumes of 1M sodium hydroxide. The effluent was tested for the presence of chloride ions by adding a few drops of 1% (w/v) silver nitrate to a sample of the effluent which had been acidified with concentrated nitric acid. The absence of a white precipitate indicated that conversion was complete.

The resin was then washed with 4 volumes of double distilled water, until the pH fell to less than pH 9, followed by 2 volumes of 1M acetic acid until the pH approached pH 2. After rinsing with double distilled water until the pH was greater than pH 5 the resin was ready for use.

Dowex AG 1 x 2, in the acetate form, was used to purify L -[U-$^{14}$C] glutamate (Section 4.1.1) and also in the assay for GAD activity to separate the [$^{14}$C] GABA produced from the residual [$^{14}$C] glutamate (Section 4.1.2).

The used resin was regenerated as follows. The resin was washed with 3 volumes of 1M hydrochloric acid to remove the glutamate followed by 3 volumes of double distilled water. Conversion of the resin to the acetate form was carried out as described above.

3.1.3 Hydroxyapatite chromatography

Synthesis of the hydroxyapatite gel (Tiselius et al 1956)

Equal volumes (1 l) 0.5M disodium hydrogen orthophosphate and 0.5M calcium chloride solutions were pumped (15 ml/minute) into a 3 l beaker and mixed by stirring slowly (80 rpm) with a large stirrer bar. When precipitation was complete the precipitate was allowed to settle

completely before the supernatant, which was slightly cloudy, was removed. The precipitate was washed four times with 1.5 l double distilled water, allowing complete settling each time.

After washing, the contents of the beaker were made up to 2 l with double distilled water and 50 ml of 40% (w/v) sodium hydroxide, which had been freshly prepared, was added stirring slowly. The contents of the beaker were heated to boiling over a 45 minute period then boiled gently for a further hour stirring continuously at 80 rpm. The gel was allowed to settle for 5 minutes, the turbid supernatant removed and the gel washed four times with 2 l double distilled water, stirring for 5 minutes then allowing to settle for 5 minutes (no heating). After the fourth wash the supernatant was removed and 2 l 0.01M sodium phosphate, pH 6.8, was added to the gel and the mixture heated to boiling, stirring gently. The gel was immediately removed from the heat and allowed to settle for 5 minutes before the supernatant was removed. This was repeated twice more, boiling for 15 minutes, then a further two times adding 2 l 0.001M sodium phosphate, pH 6.8, and boiling for 15 minutes. After it had been boiled for the fifth time the gel was ready for use. The quantities used produced approximately 300 ml gel which will remain effective for about 1 year when stored in 0.002M sodium phosphate, pH 6.8, at 4°C.

Before it was used for column chromatography the hydroxyapatite gel was washed into 0.002M potassium phosphate, pH 7.4 and mixed with preswollen cellulose which had been equilibrated in the same buffer. Slurries of the hydroxyapatite and of the cellulose were left to settle for between 6 and 9 hours at 4°C before the settled gels were mixed together in the ratio 1:1 (v/v). Whilst the column is being packed the slurry yet to enter the column must be continuously mixed to ensure an even distribution of the hydroxyapatite and cellulose. The addition of cellulose allowed rapid elution (80 ml/hour) of the columns.

Experiments using the hydroxyapatite column are described in Section 5.1.

## 3.2 Polyacrylamide gel electrophoresis

### 3.2.1 Non denaturing

Non denaturing polyacrylamide gel electrophoresis (using both slab and disc gels) was carried out using a single gel of uniform pore size and a discontinuous buffer system similar to that described by Gordon and Louis (1967). Using this system the sample is concentrated and the proteins stack according to their charge as they enter the gel. Since band sharpening occurs after all the constituents of the protein mixture have entered the gel (i.e. after some degree of separation has occurred) for good results the sample must be applied as a small volume of a concentrated solution.

Samples, concentrated as described in Section 3.3, were mixed with 10% glycerol, containing bromophenol blue as the tracking dye, and loaded onto 5% polyacrylamide disc or slab gels. The constituents of the gel were 5% acrylamide (w/v), 0.25% bis acrylamide (%) , 0.3% polyacrylamide, 0.05% Temed (v/v), 2 mM ammonium persulphate and 2 mM EDTA. The gels were buffered with 0.37M Tris phosphate, pH 8.8. The electrode buffer was 0.05M sodium borate containing 2 mM EDTA, pH 9.2.

For disc gels electrophoresis was carried out at room temperature, 3 mA/gel (constant current) until the tracking dye was 1 cm from the bottom of the gel.

For slab gels electrophoresis was carried out at room temperature, 150 V (constant voltage) until the tracking dye was approximately 2 cm from the bottom of the gel. The electrode buffer in the upper reservoir was changed once, halfway through the electrophoresis, since electrolysis resulted in a change in pH which might affect the electrophoresis.

The gels were fixed and stained as described in Section 3.2.3.

#### 3.2.1.1 'Activity' gels

These were 5% non denaturing disc or slab gels prepared as described above. However, before loading the samples, the gels were prerun, at 4°C, for 1.5 hours at 3 mA/gel (disc gels) or for 3 hours at 150 V (slab gels) with 0.37M Tris phosphate containing 2 mM EDTA, pH 8.8, as electrode buffer to remove oxidising agents. The Tris phosphate buffer was replaced with sodium borate electrode buffer and electrophoresis carried out as described previously for non denaturing gels but at 4°C rather than room temperature.

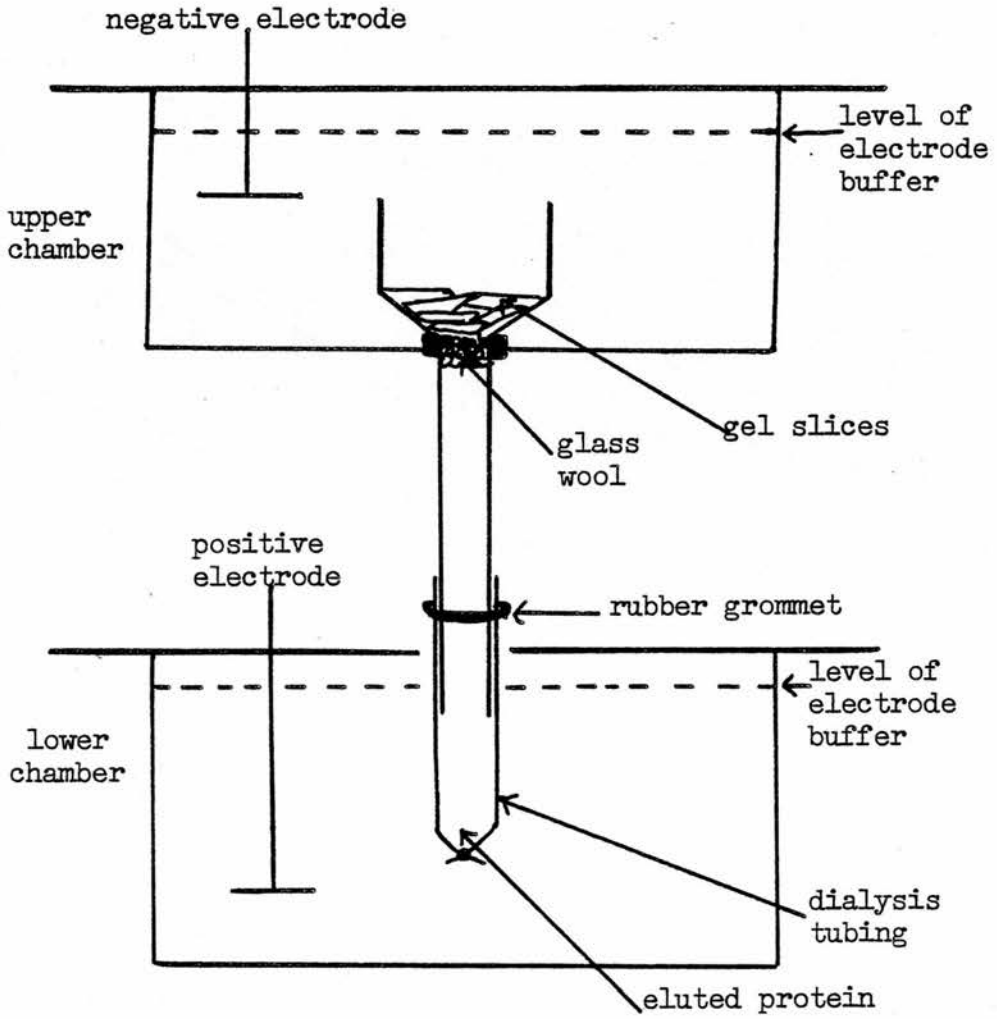



Figure 3.3 The apparatus used to elute proteins from slices of polyacrylamide gel. See text, section 3.2.1.2.2. for description and experimental details.

3.2.1.2 Elution of proteins from 'activity' gels

3.2.1.2.1 Elution into standard buffer

Unstained 'activity' disc gels were cut into slices which were 3 mm thick. Corresponding regions from several gels were pooled, finely macerated and eluted into 400 µl of standard buffer, at 4°C, for 4 hours or overnight. Samples were taken from the elution buffer and assayed for GAD activity as described in Section 4.1.2.

3.2.1.2.2 Elution by electrophoresis

The $R_F$ values ($R_F$ = relative mobility of the protein with respect to the tracking dye bromophenol blue) for proteins in a non denaturing polyacrylamide slab gel were determined after fixing and then staining with Coomassie Blue (Section 3.2.3.1). These $R_F$ values were used to estimate the position of the proteins in equivalent unstained 'activity' slab gels. The appropriate areas of the gel were excised, equivalent regions pooled and the proteins eluted using the apparatus shown in Figure 3.3.

Glass tubes, of the same diameter as those used for disc electrophoresis, were modified by the addition of a chamber to contain slices of gel. As many as 4 of these tubes were fitted into a standard, 8 place, disc gel electrophoresis apparatus so that gel slices taken from 4 different areas could be eluted simultaneously. Visking dialysis tubing (1 - 8/32") was tightly fastened to the tubes by means of a rubber grommet.

The whole apparatus was filled with sodium borate electrode buffer (see Section 3.2.1) and the protein in the gel slices eluted by electrophoresis at 3 mA/tube (constant current) at 4°C for 4 to 6 hours or overnight. The eluted protein was assayed for GAD activity as described in Section 4.1.2.

3.2.2 Denaturing

Whereas non denaturing polyacrylamide gel electrophoresis separates proteins on the basis of charge, SDS polyacrylamide gel electrophoresis separates proteins primarily on the basis of their $M_r$ (Shapiro *et al* 1967). In general, over a wide range of $M_r$ there is a linear relationship between log $M_r$ of a protein and its $R_F$ value (Shapiro *et al* 1967; Weber & Osborn 1969). There are some exceptions to this caused by anomalous binding of SDS, for example to glycoproteins or to proteins of a very acidic nature.

Table 3.2. $M_r$ standards for denaturing polyacrylamide gel electrophoresis

| Protein | Source | $M_r$ | Reference |
|---|---|---|---|
| Cholera toxin (B subunit) | *Vibrio cholerae* | $11.6 \times 10^3$ | Nakashima *et al* (1976) |
| RNAase | Bovine pancreas | $13.7 \times 10^3$ | Weber and Osborn (1969) |
| Cholera toxin ($A_1$ polypeptide) | *Vibrio cholerae* | $22 \times 10^3$ | Lai *et al* (1976) |
| Immunoglobulin G Light chain (IgG, L) | Bovine | $23.5 \times 10^3$ | Weber and Osborn (1969) |
| Lactate dehydrogenase (LDH) | Bovine heart | $36 \times 10^3$ | Weber and Osborn (1969) |
| Ovalbumin | Egg | $43 \times 10^3$ | Weber and Osborn (1969) |
| Immunoglobulin G Heavy chain (IgG, H) | Bovine | $50 \times 10^3$ | Weber and Osborn (1969) |
| Serum albumin (BSA) | Bovine | $68 \times 10^3$ | Weber and Osborn (1969) |
| Phosphorylase *b* | Rabbit muscle | $94 \times 10^3$ | Cohen *et al* (1971) |

This relationship can be used to determine the $M_r$ of proteins of unknown size by using a number of well characterised marker proteins, of known $M_r$, to calibrate each gel.

Table 3.2 lists the proteins which were used as marker proteins to calibrate 11% and 15% (w/v) polyacrylamide SDS/urea gels. Semi logarithmic plots of $R_F$ versus log $M_r$ obtained by using a selection of those proteins in 11% and 15% polyacrylamide gels are shown in Figures 3.4 and 3.5 respectively. The line of best fit was calculated using the nonparametric method of Nimmo and Atkins (1979). Such plots were used to calculate the $M_r$ of the polypeptides present in the most purified GAD preparations.

Urea was added to the sample incubation buffer and gel buffer to prevent reaggeregation of the polypeptides. In addition it has been shown that if urea is present the range of $M_r$ over which the semi logarithmic relationship with $R_F$ is linear can be extended to include proteins of very low $M_r$ (Swank & Munkres 1971).

Denaturing polyacrylamide slab gel electrophoresis was carried out using 11% and 15% (w/v) polyacrylamide slab gels, containing SDS and urea, and a discontinuous buffer system similar to that used by Laemmli (1970) except that urea was incorporated in both the gel buffer and the sample incubation buffer.

Polyacrylamide gels (final concentrations) were either 11% acrylamide (w/v), 0.15% bis acrylamide (w/v) or 15% acrylamide (w/v), 0.2% bis acrylamide (w/v), containing 0.075% Temed (v/v), 2 mM ammonium persulphate, 0.1% SDS (w/v) and 4M urea buffered with 0.37M Tris HCl, pH 8.8.

The electrode buffer was 0.02M Tris/0.2M glycine, pH 8.3, containing 0.1% SDS (w/v).

The sample was mixed 4:1 (v/v) with the sample incubation mixture (0.12M Tris HCl containing 4% SDS (w/v), 2% B mercaptoethanol (v/v), 8M urea, pH 6.8) and incubated at about 60$^{o}$C for 15 to 30 minutes. After incubation the sample was made 10% (v/v) with respect to glycerol and, with bromophenol blue as tracking dye, was loaded onto the gel. Electrophoresis was carried out at 150 V (constant voltage) until the tracking dye was approximately 2 cm from the bottom of the gel. The gels were fixed and stained as described in Section 3.2.2.1.

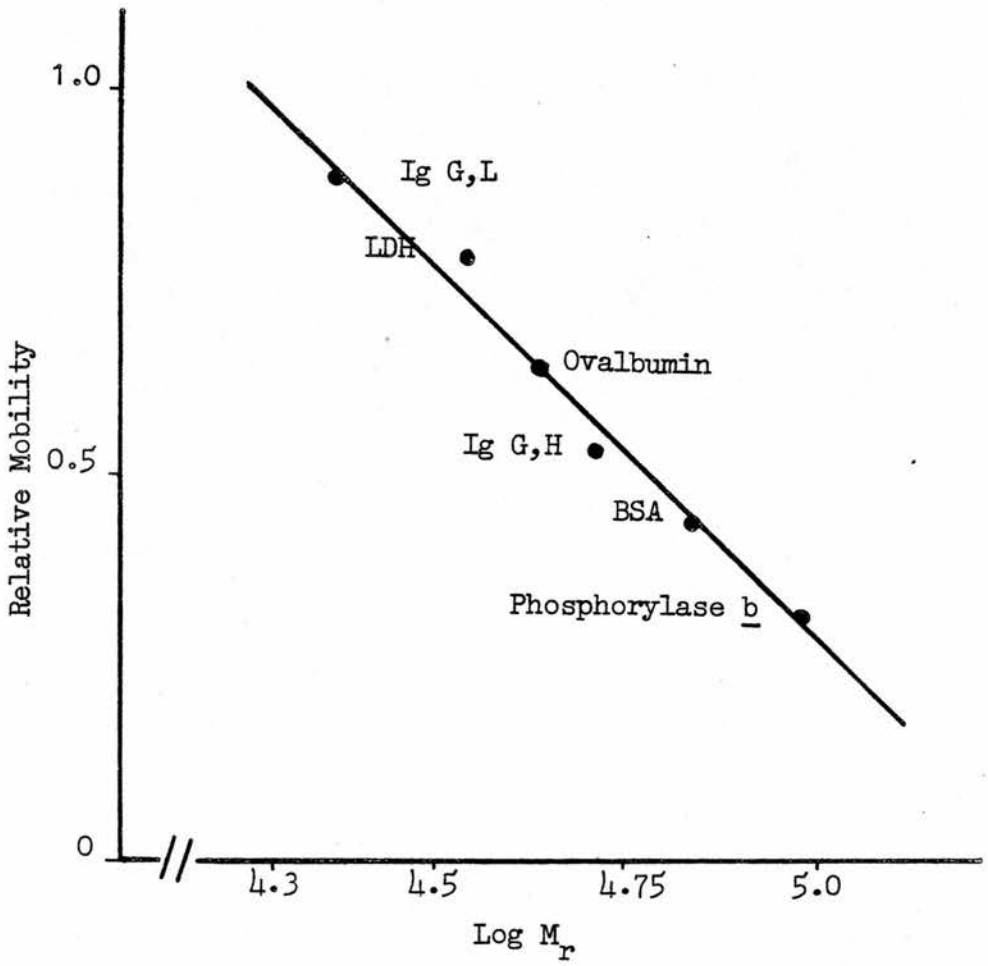



Figure 3.4 A graph of relative mobility (in an 11% polyacrylamide slab gel containing SDS and urea ) versus log $M_r$ for some of the proteins listed in Table 3.2. Details of the composition of the gel can be found in section 3.2.2. The best straight line was calculated using the method of Nimmo and Atkins (1979).

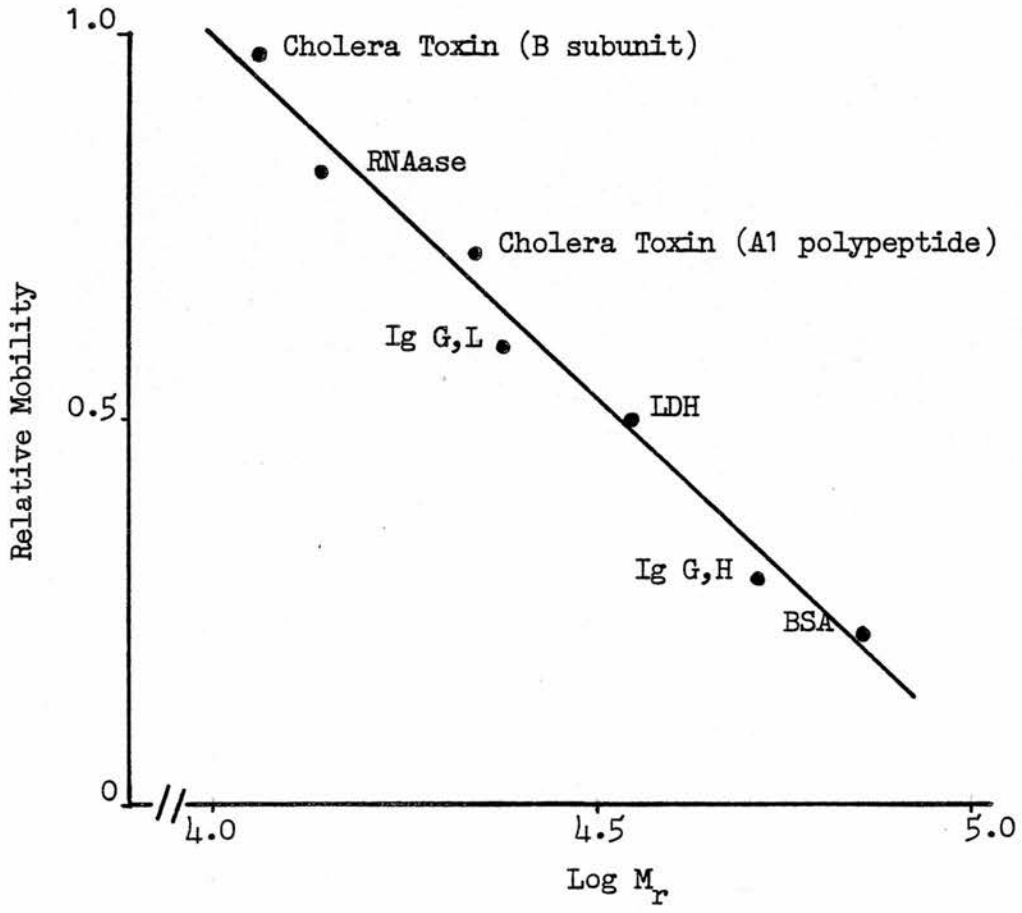



Figure 3.5 A graph of relative mobility (in a 15% polyacrylamide slab gel containing SDS and urea ) versus log $M_r$ for some of the proteins listed in Table 3.2. Details of the composition of the gel can be found in section 3.2.2. The best straight line was calculated using the method of Nimmo and Atkins (1979).

### 3.2.3 Staining of proteins in polyacrylamide gels

#### 3.2.3.1 Coomassie Brilliant Blue

Prior to staining with Coomassie Blue the protein bands were fixed by soaking the gel for 30 minutes in a mixture 50% methanol/10% acetic acid (v/v) in water.

Coomassie Blue stain was made by dissolving 1.25 g Coomassie Brilliant Blue R in a mixture of 454 ml 50% methanol (v/v) in water and 46 ml glacial acetic acid. After stirring vigorously overnight the stain was filtered through Whatman number 1 chromatography paper before use.

The gels were destained by shaking gently at 37°C in an excess of destain which consisted of 7.5% glacial acetic acid and 5% methanol (v/v) in water.

#### 3.2.3.2 Silver staining

Silver staining of proteins in polyacrylamide gels was carried out using the method of Wray et al (1981). The method can be used to detect proteins at the nanogram level and may be used before or after staining with Coomassie Blue.

The polyacrylamide gel was soaked overnight in 50% (v/v) methanol in water. The stain was prepared as follows. Silver nitrate (0.8 g) was dissolved in 4 ml double distilled water and this was added dropwise, with constant stirring, to a solution which consisted of 21 ml 0.09M sodium hydroxide and 1.4 ml 14.8M ammonium hydroxide. The stain was then made up to 100 ml with water. This solution must be used immediately. The gel was stained in this solution for 15 minutes whilst being shaken gently. After it had been washed for 5 minutes in double distilled water the gel was soaked in freshly made developer solution until bands started to appear.

The developer solution was a mixture of 2.5 ml 1% (w/v) citric acid and 0.25 ml 38% (w/v) formaldehyde made up to 500 ml with double distilled water. Bands should appear after approximately 10 minutes. The gel was washed in double distilled water and placed in 50% (v/v) methanol to prevent further development. The gel was stored in 25% (v/v) methanol/10% (v/v) acetic acid.

## 3.3 Concentration of protein

### 3.3.1 Ultrafiltration

The Amicon ultrafiltration system was used to concentrate samples containing GAD. The ultrafiltration system, using a stirred cell equipped with a DIAFLO PM 30 membrane, was assembled and operated according to the manufacturers'instructions. Samples, in standard buffer, were concentrated at 4°C under nitrogen. This method was used primarily to prepare samples for SDS/urea polyacrylamide gel electrophoresis since ultrafiltration always resulted in large losses of GAD activity.

### 3.3.2 Using dry Sephadex G25

Solutions of high molecular weight, such as proteins, can be concentrated by the addition of dry Sephadex G25. Water and small ions are absorbed into the swelling Sephadex beads whilst the proteins remain in the external solution. In this way a protein solution can be concentrated whilst the pH and ionic strength remain the same (Flodin et al 1960).

Sample concentration using dry Sephadex G25 was carried out at 4°C. Dry Sephadex G25 was added, in the proportions 1 g gel per 4 ml solution, to the sample contained in a small glass column. After thorough mixing the gel was allowed to swell for 10 minutes before the proteins were eluted. Blue dextran (0.2%) was added to the first 10% of the elution buffer as an indicator of the end of the sample. In this way a 2 to 3 fold concentration was achieved. If the process was repeated a five fold concentration could be obtained with only a negligible reduction of total GAD activity, the specific activity remained constant.

Samples containing GAD activity were concentrated in this manner when subsequent experimental procedures required a high level of GAD activity e.g. for gel filtration using Sephadex G200 (Sections 5.1 and 5.3) or for non denaturing polyacrylamide gel electrophoresis on 'activity' gels followed by elution of the protein bands (Section 5.2) and assay for GAD activity (Section 4.1.2).

## 3.4 Paper electrophoresis

Samples (1 µl to 15 µl) were applied, at 2 cm intervals, to the midline of a strip of Whatman number 1 chromatography paper, 23 cm long and up to 10 cm wide. Electrophoresis was carried out at 200 V for 20 to 30 minutes in pyridine-acetic acid buffer, pH 6.5. This buffer

was prepared by mixing 100 ml pyridine, 4 ml glacial acetic acid and 1900 ml double distilled water.

Amino acids were stained by spraying the dried paper with a solution of 0.2% (w/v) ninhydrin in acetone and drying in the oven at 70°C to 80°C.

### 3.5 Scintillation counting

For non-aqueous samples or aqueous samples up to and including 0.5 ml, toluene-methoxyethanol scintillation fluid was used. This consisted of 6 g PPO per litre of a 1:1 (v/v) mixture of toluene and methoxyethanol.

For aqueous samples up to and including 5 ml, toluene-Triton scintillation fluid was used. This consisted of 6 g PPO (see abbreviations) per litre of a 2:1 (v/v) mixture of toluene and Triton X100.

Samples were counted on a Searle MK III liquid scintillation system which calculates dpm by the sample channels-ratio method or on a Packard Tri-carb 460 C liquid scintillation system which calculates dpm using both the sample channels-ratio method and an external standard (Peng 1977).

### 3.6 Assay methods

#### 3.6.1 Protein assay

The method of Lowry et al (1951) was used for all determinations of protein concentration. Bovine serum albumin was used as a standard. The eluant from chromatography columns was monitored for protein by measuring the absorbance at 280 nm.

#### 3.6.2 GAD assay

The assay used to measure GAD activity is described in Section 4.1.2.

#### 3.6.3 Phosphate assay

The sample was made up to 1 ml with distilled water and 3 ml of 1M perchloric acid, 0.5 ml of concentrated perchloric acid and 1 ml of 2.5% ammonium molybdate were added. The solution was mixed thoroughly. After heating at 60°C for 5 minutes 1 ml 0.1M ascorbic acid was added, the solution mixed throughly and heated at 60°C for 15 minutes. The samples were read against a reagent blank (containing no phosphate) on a Pye Unicam SP6-500 spectrophotometer at 650 nm. Standards which contained between 0 and 1 μmol of inorganic phosphate were used to construct a standard curve.

3.6.4 GABA-T assay

GABA-T activity in samples was estimated by measuring the amount of succinic semialdehyde (SSA) produced by GABA-T during the assay (Taberner et al 1972). SSA was measured using the colorimetric method described by Taberner et al (1972) which involves reacting the SSA with 3-methyl-2-benzothiazotrione hydrazone (MBTH) to form a stable coloured product.

3.6.4.1 Tissue preparation and GABA-T incubation

A tissue homogenate, 1:10 (w/v), in 0.32M sucrose was either sonicated or frozen to -40°C and thawed to disrupt the mitochondria. A 100 µl sample (containing about 1 mg protein) was added to 400 µl of the GABA-T incubation buffer so that the final incubation concentrations were 50 mM GABA, 10 mM 2-oxoglutarate, 0.1 mM PLP buffered with 100 mM sodium phosphate, pH 8.2. After incubation for 1 hour at 37°C the reaction was stopped by the addition of 15 µl concentrated perchloric acid. The precipitated protein was removed by centrifugation for 1 minute in a Beckman microfuge at approximately 10 000 g and 75 µl of the supernatant assayed for SSA. Controls consisted of an enzyme blank to which perchloric acid was added before incubation at 37°C.

3.6.4.2 Reaction of SSA with MBTH

The MBTH colour reaction was linear over the range 0.01 to 0.1 µmol SSA, Taberner et al (1972) and as shown by the standard curves obtained in this work (see Figure 3.6). A sample volume of 75 µl was chosen since it gave values on the linear portion of the SSA standard curve.

The sample to be analysed was added to 0.4 ml of a 1% (w/v) aqueous solution of MBTH (freshly prepared since the solution is unstable) and mixed. The solution was allowed to stand for 5 minutes at room temperature, heated at 100°C for 3 minutes then cooled for 6 minutes before the addition of 2 ml of a 0.2% (w/v) aqueous ferric chloride solution. The mixture was shaken then after 5 minutes the volume was made up to 6 ml with acetone. After 40 minutes (when the colour development was complete) the samples were read at 650 nm versus the controls (samples taken from enzyme blanks to which perchloric acid had been added before incubation at 37°C) on a Pye Unicam SP6-500 spectrophotometer.

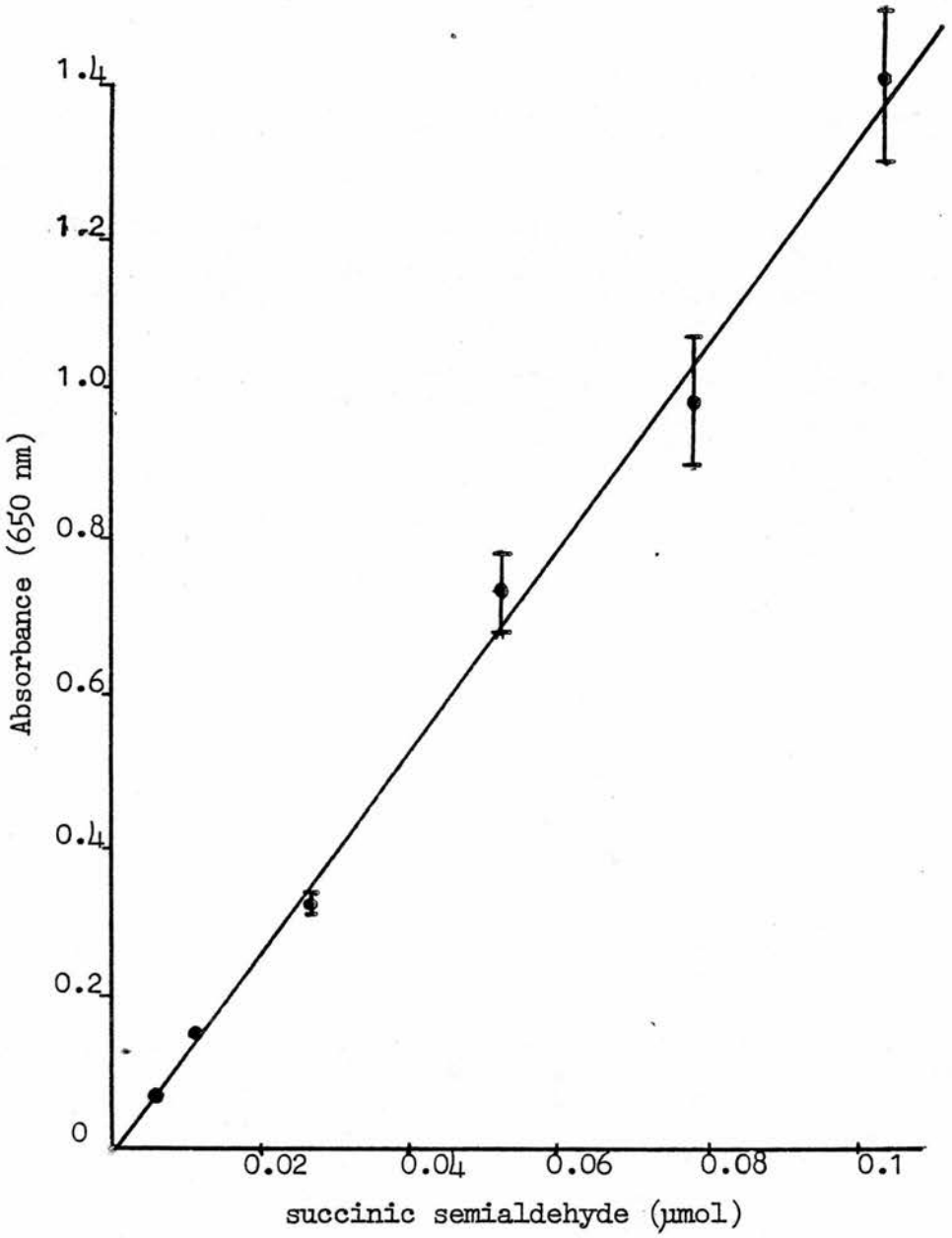



Figure 3.6 Standard curve for the MBTH colour reaction in the GABA-T assay. See section 3.6.4. Each value is a mean ± s.e.m. , n=4

3.6.5 Measurement of high affinity uptake of [$^3$H] GABA

The high affinity uptake of [$^3$H] GABA into chick brain tissue homogenates was measured using a procedure modified from that described by Bondy and Purdy (1977). The standard incubation medium consisted of Krebs-Ringer buffer which was 121 mM NaCl, 4.0 mM KCl, 1.3 mM $CaCl_2.H_2O$, 1.2 mM $MgSO_4.7H_2O$, 0.015 mM EDTA, 20 mM glucose, 1 mM ascorbic acid, 40 mM Tris HCl, pH 7.6, and contained 10 uM AOAA (see abbreviations) (an inhibitor of GABA-T activity, Wallach 1961) and 0.01 uM [2,3-$^3$H] GABA (specific radioactivity = 740 kBq/nmol). This medium was gassed with 95% $O_2$ - 5% $CO_2$ and 0.9 ml aliquots were dispensed into small stoppered glass tubes. After preheating at 37°C the incubation was started by the addition of 0.05 ml of a brain tissue homogenate, 1:100 (w/v) in 0.32M sucrose. After 5 minutes 0.05 ml ice-cold liver homogenate (1:10 w/v in 0.32M sucrose), which does not have a high affinity uptake system for GABA (Goodchild & Neal 1973) was added as carrier protein, quickly mixed and the incubation mixture emptied on to a Whatman GFC (2.5 cm diameter) filter disc in a filtration tower. The incubation medium was drawn through the filter by applying a vacuum and the filter washed with 3 x 5 ml of ice cold isotonic saline.

Each incubation was carried out in triplicate with a parallel set of control incubations at 0°C to account for neurotransmitter binding which was not energy dependent. The amount of radioactivity on the filter discs was determined by immersing the discs in 5 ml toluene-methoxyethanol scintillation fluid followed by liquid scintillation counting as described in Section 3.5. The filter discs were immersed in scintillation fluid for 90 minutes before scintillation counting with occasional shaking, to release the radiolabel.

Any loss of homogenate through the filter or loss of [$^3$H] GABA from the tissue caused by the filtration procedure would lead to an underestimation of the amount of [$^3$H] GABA accumulated by the homogenate. However, control experiments, using centrifugation to separate the homogenate tissue from the incubation medium, produced similar results to those obtained using the filtration method (J. Haywood, unpublished data).

## SECTION 4: L-GLUTAMATE DECARBOXYLASE (GAD)

### 4.1 GAD assay

The assay used to measure L-glutamate decarboxylase (GAD EC.4.1.1.15) activity was a modification of that used by Kanazawa et al (1976). GAD activity was determined by measuring the amount of radioactive GABA produced from L-[U-$^{14}$C]glutamate. The [$^{14}$C] GABA was separated from the residual [$^{14}$C] glutamate by ion exchange chromatography.

Commercial L-[U-$^{14}$C] glutamate contains radioactive impurities, some of which would be eluted from the ion exchange column with the [$^{14}$C] GABA and produce artefactually high blank values. The [$^{14}$C] glutamate was therefore, partially purified by removing those substances bearing a similar charge to GABA.

#### 4.1.1 Purification of [$^{14}$C] glutamate

The [$^{14}$C] glutamate was purified by ion exchange chromatography using a Dowex AG 1-X2 (100-200) mesh column (0.6 cm x 3 cm) in the acetate form. The conversion of Dowex from the chloride to the acetate form is described in Section 3.1.2.2. A 1 ml (1.85MBq) aliquot of [$^{14}$C] glutamate was applied to the column which was then washed with 15 ml double distilled water to elute any uncharged or positively charged impurities. This was followed by 15 ml of 0.1M phosphoric acid to elute the [$^{14}$C] glutamate. Fractions, 0.5 ml, were collected throughout. Any radioactive fractions eluted with water were discarded and only those fractions eluted with phosphoric acid and containing the highest radioactivity were collected. These fractions were pooled and evaporated to dryness in a vacuum dessicator.

The [$^{14}$C] glutamate was resuspended in 1 ml of potassium phosphate buffer containing unlabelled glutamate so that the final glutamate concentration was 250 mM. The specific radioactivity ranged from 6.66 kBq/μmol to 7.4 kBq/μmol depending on the efficiency with which the [$^{14}$C] glutamate was recovered. The phosphate concentration in the buffer was calculated, taking into account the phosphate ions already present due to the elution with phosphoric acid, so that the phosphate concentration was 500 mM. The pH was adjusted to pH 7.4 using 10M potassium hydroxide.

Paper electrophoresis of the purified [$^{14}$C] glutamate was carried out to establish that all the radioactivity comigrated with glutamate (see Figure 4.1). Small amounts of unpurified and partially purified glutamate (4 µl, about 400 000 dpm and 2.5 µl, about 200 000 dpm respectively) were loaded in duplicate with a GABA standard (5 µl of a 10 mM GABA solution) on to the centre of a strip of Whatman no. 1 chromatography paper. Section 3.4.1 describes the buffers and voltage used.

After electrophoresis the strip was divided, one half was treated with 0.2% ninhydrin (w/v in acetone) and the other half cut into 1 cm squares. These were immersed in 5 ml toluene-methoxyethanol scintillation fluid and left for 90 minutes before liquid scintillation counting (see Section 3.5).

4.1.2 Measurement of [$^{14}$C] GABA produced by GAD

The purified [$^{14}$C] glutamate in potassium phosphate, pH 7.4 (see previous section) was added, in the proportions 1:4 (v/v), to a solution which was 2.5 mM with respect to both pyridoxal-5-phosphate (PLP) and dithiothreitol (DTT). A 5 µl aliquot of this assay mixture was added to 5 µl of the enzyme sample to give final concentrations in the assay as follows: [$^{14}$C] glutamate, 25 mM (specific radioactivity between 6.66 kBq/µmol and 7.4 kBq/µmol); potassium phosphate, 50 mM; PLP, 1 mM; DTT, 1 mM; pH 7.4.

After a 30 minute incubation at 37°C each assay was terminated by adding 0.4 ml ice cold double distilled water and the tubes transferred to ice. Each sample was then applied to a column (0.6 cm x 1.5 cm) of Dowex acetate. The [$^{14}$C] GABA which had been produced was washed through the column with 1.5 ml ice cold double distilled water and collected in scintillation vials. Toluene-Triton scintillation fluid (12 ml) was added and the radioactivity counted (see Section 3.5).

4.1.3 Paper electrophoresis of the products of the GAD assay

The products of the GAD assay were analysed by paper electrophoresis (see Section 3.4.1) to ascertain whether the radioactivity in the sample, after ion exchange chromatography, was due to [$^{14}$C] GABA. Electrophoresis of the products of the reaction without prior ion exchange chromatography was carried out for comparison.

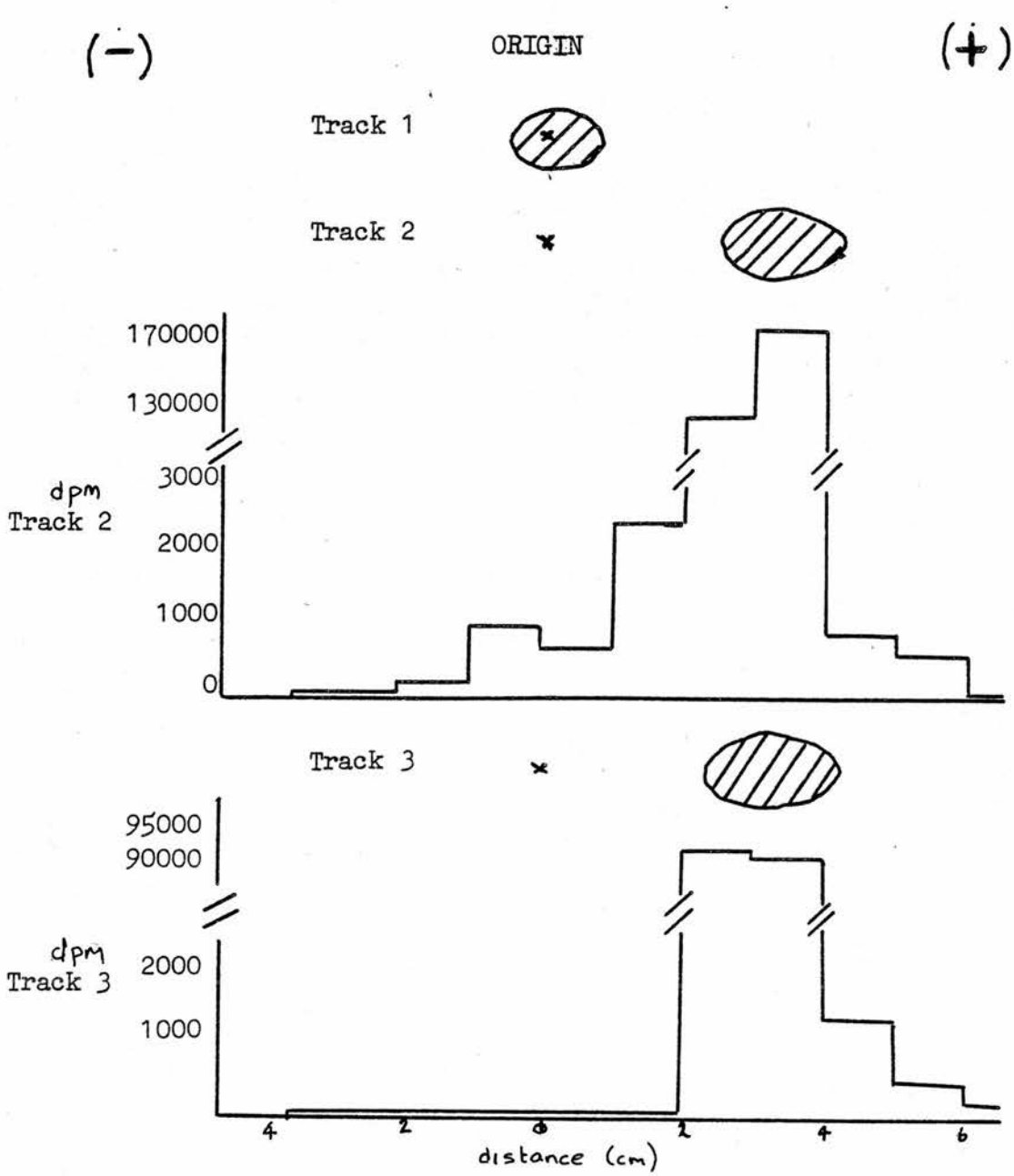



Figure 4.1 Comparison between unpurified and partially purified [$^{14}$C] glutamate by electropherisis, See section 4.1. Track 1 is a GABA standard to compare relative mobilities of glutamate and GABA under these conditions. Track 2 is 4µl of unpurified [$^{14}$C]glutamate (400000 dpm ). Track 3 is 2.5 µl of partially purified [$^{14}$C] glutamate (200000 dpm ). Conditions of electrophoresis are described in section 3.4.1.

Incubations were set up as follows:

(a) Four tubes each containing 10 µl of a chick brain supernatant (homogenate 1:10 (w/v) in standard buffer and centrifuged for 1 hour at 100 000 $g_{av}$, 4°C) and 10 µl GAD assay mixture, incubated for 1 hour at 37°C.

(b) Two tubes, each containing 5 µl chick brain supernatant and 5 µl GAD assay mixture, incubated for 1 hour at 37°C.

(c) Four blanks, each containing 5 µl boiled chick brain supernatant and 5 µl GAD assay mixture, incubated for 1 hour at 37°C.

The two incubations from (b) and two of the blanks from (c) were processed as described in 4.1.2 and counted to determine the amount of [$^{14}$C] GABA produced.

Two of the incubations from (a) and one of the blanks from (c) were passed through ion exchange columns (as described in Section 4.1.2) and the eluants evaporated to dryness in a vacuum dessicator. Each sample was resuspended in 20 µl of distilled water and 5 ul of each was applied, in duplicate, to a strip of Whatman no. 1 chromatography paper with 5 µl 10 mM glutamate and 5 µl 10 mM GABA as standards (see Figure 4.2).

The remaining two incubations from (a) and a blank were evaporated to dryness, without prior ion exchange chromatography, then applied to chromatography paper exactly as described above (see Figure 4.2).

After electrophoresis the two paper strips were cut in half and, in each case, one half was stained with ninhydrin whilst the other half was cut into 1 cm squares which were placed in scintillation vials. Toluene-methoxyethanol scintillation fluid (5 ml) was added and the radioactivity counted so that the position of the radioactivity could be determined in relation to the position of the glutamate and GABA.

Although this was not done quantitatively the electrophoresis showed that [$^{14}$C] GABA was the only labelled product of the GAD reaction. Figure 4.2, track 3 shows GABA comigrating with the radioactivity when the incubation contained active GAD but no GABA (or corresponding radioactivity) when the incubation contained heat inactivated GAD, track 4. In addition it was shown that the ion exchange columns used in the assay completely retained the [$^{14}$C] glutamate present in the assay, Figure 4.2B, since the only radioactivity detected was due to GABA.

Figure 4.2. Electrophoresis of the products of the GAD assay

See Section 4.1.3 for details.

A = before ion exchange chromatography

B = after ion exchange chromatography

In each case track 1 = glutamate standard

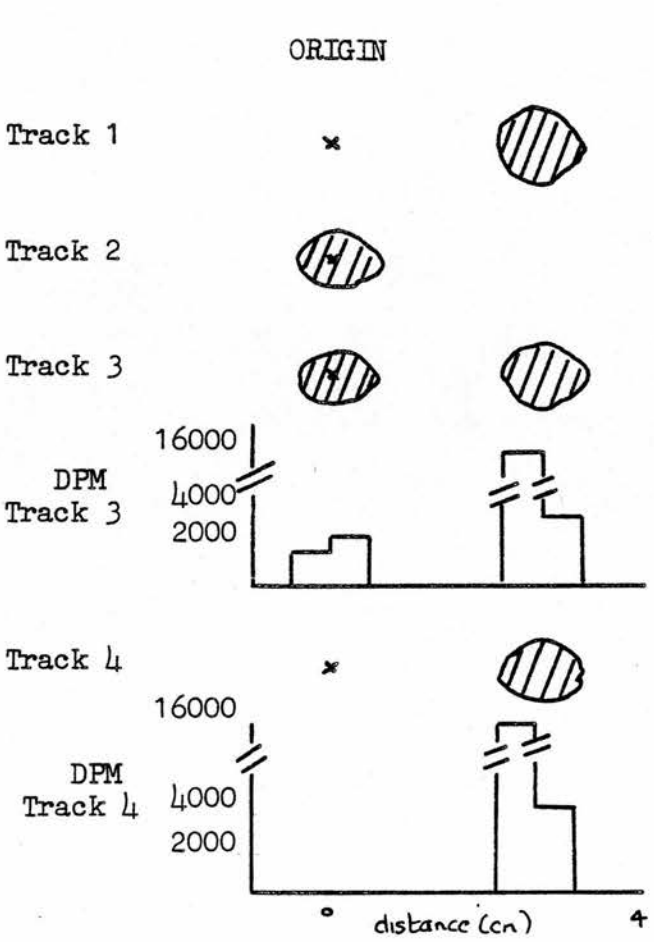
track 2 = GABA standard

track 3 = incubation contained active GAD

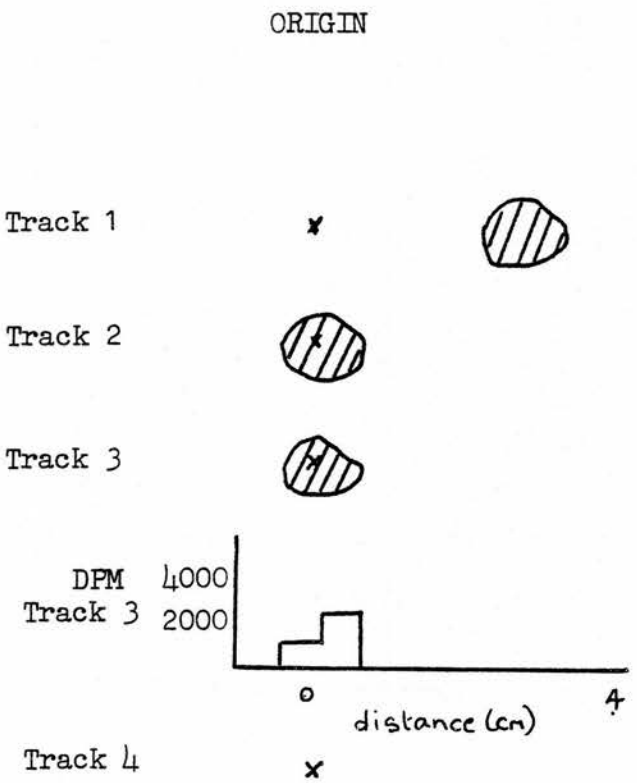
track 4 = blank i.e., incubation contained heat inactivated GAD.

Samples (5 µl) were spotted onto strips of Whatman no. 1 chromatography paper and electrophoresis was carried out as described in Section 3.4.1.

A


(−)
ORIGIN
(+)
Track 1
Track 2
Track 3
16000
DPM
Track 3
4000
2000
Track 4
16000
DPM
Track 4
4000
2000
0
distance (cm)
4

B


(−)
ORIGIN
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Track 2
Track 3
DPM
Track 3
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2000
0
distance (cm)
4
Track 4

## 4.2 Characteristics of the GAD assay

The following experiments all used chick whole brain supernatants, prepared by centrifuging an homogenate at 100 000 $g_{av}$, 4°C, for 1 hour, as a source of GAD activity.

### 4.2.1 Linearity of GABA production versus time

Experiments were carried out in which the GAD assay was terminated after several different time periods to determine how long the reaction rate remained linear. The assay was performed as described in Section 4.1.2 using chick brain cytosol prepared from an homogenate, 1:10 (w/v) in standard buffer (approximately 15-20 µg protein/assay), as a source of enzyme activity. The assay was halted after 0, 10, 20, 30, 45 and 60 minutes and the amount of [$^{14}$C] GABA produced was determined (Section 4.1.2). The data shown in Figure 4.3 were taken from 5 experiments. The best straight line was calculated using the nonparametric method of Nimmo and Atkins (1979). Under the assay conditions used the production of GABA was linear for 1 hour at 37°C, consequently 30 minutes was chosen as a suitable incubation period for the assay.

### 4.2.2 The effect of varying protein concentration on the production of GABA

The total amount of protein in the assay was varied from 1.3 µg/assay to 45.4 µg/assay. This was achieved by serial dilution, in standard buffer, of chick brain cytosol prepared from an homogenate, 1:3 (w/v) in standard buffer. Using samples of diluted cytosol as a source of GAD activity the assay was carried out as described in Section 4.1.2. Figure 4.4 shows the pooled results from three experiments. The best straight line, considering all the points, was calculated using the nonparametric method of Nimmo and Atkins (1979). GAD activity was proportional to protein concentration, under these assay conditions, when the protein concentration was varied from 0.26 mg/ml to 9.07 mg/ml.

### 4.2.3 pH profile

The GAD activity in chick brain cytosol (prepared from an homogenate 1:10 (w/v) was determined over a range of pH values, pH 5.5, 6.5, 7.0, 7.5, 8.5 and 9.5. Apart from the alteration of pH the assay was carried out as described in Section 4.1.2.

A plot of GAD activity versus pH is shown in Figure 4.5. The pH profile obtained shows that the pH optimum for the assay is near pH 7.

Figure 4.3. GAD activity as a function of time

Chick brain cytosol was incubated at 37°C under the conditions described in Section 4.2.1. One unit of enzyme activity is 1 nmol GABA produced/mg protein. Each value is the mean ± S.E.M., n = 5. The best straight line was calculated using the nonparametric method of Nimmo and Atkins (1979).

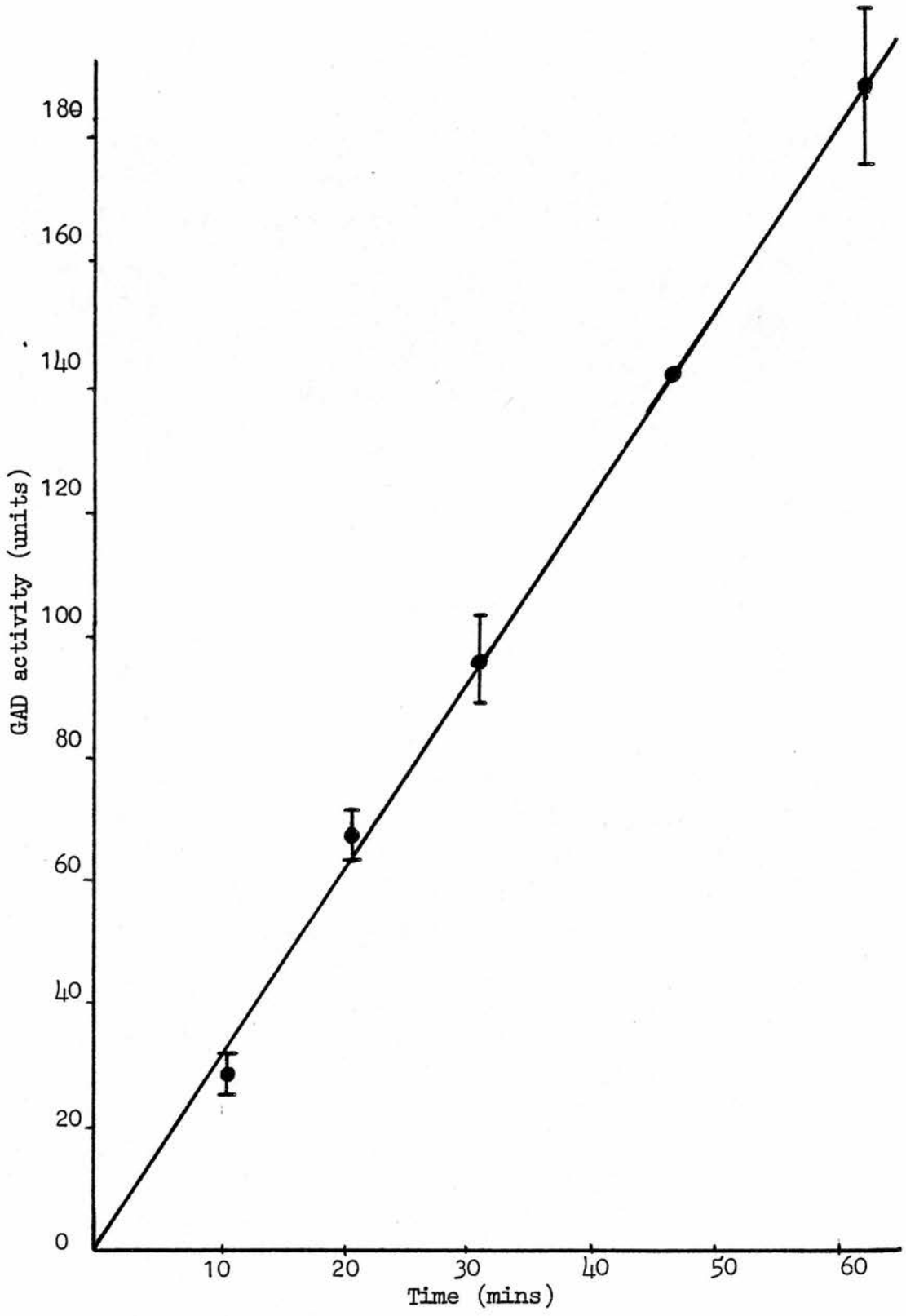
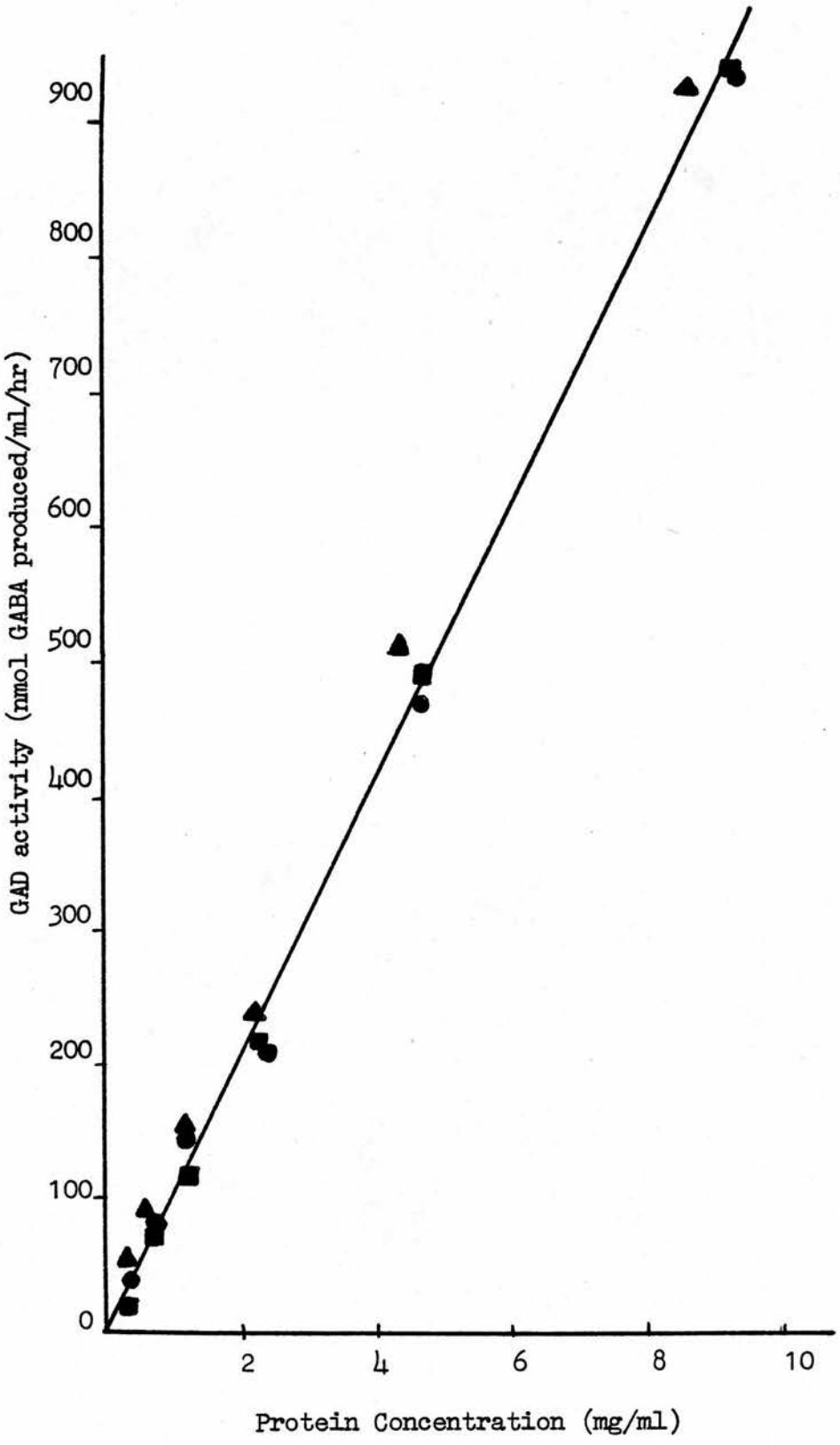

GAD activity (units)
180
160
140
120
100
80
60
40
20
0
10
20
30
40
50
60
Time (mins)

Figure 4.4. GAD activity as a function of protein concentration in the assay

Experimental details are described in Section 4.2.2. The data shown are the means from three separate experiments, each determination was in triplicate. The best straight line through all the data was calculated using the nonparametric method of Nimmo and Atkins (1979). GAD activity is expressed as nmol GABA produced/ml incubation./hr.


GAD activity (nmol GABA produced/ml/hr)
900
800
700
600
500
400
300
200
100
0
2
4
6
8
10
Protein Concentration (mg/ml)

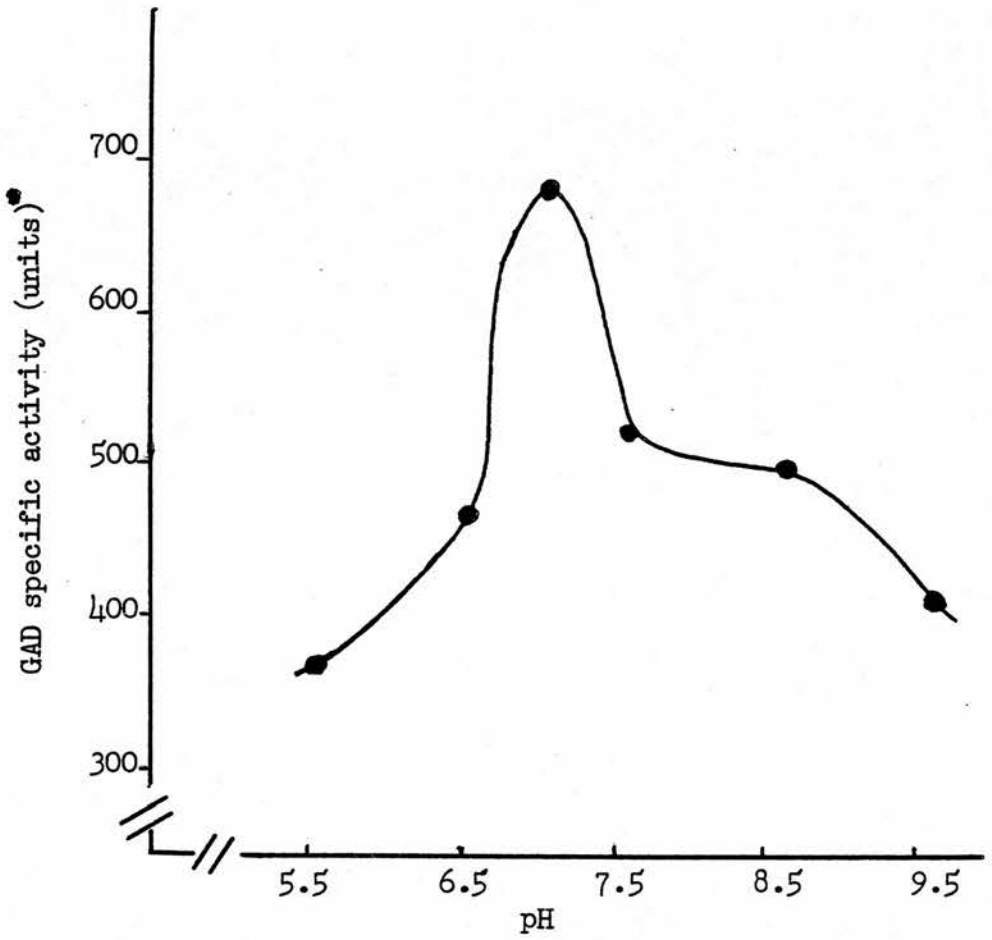



Figure 4.5 pH profile for GAD in chick brain cytosol. See section 4.2.3. One unit of specific enzyme activity = one nmol. GABA produced / mg protein / hour.

## 4.3 $K_m$ values of chick brain GAD

### 4.3.1 Apparent $K_m$ for glutamate

Since chick brain GAD could not be purified to homogeneity (see Section 5) the apparent $K_m$ for glutamate was determined using chick brain cytosol (prepared from an homogenate, 1:3 (w/v) in standard buffer) which had been desalted by gel filtration on Sephadex G25 (see Section 3.1.1.2). The assay conditions were as described in Section 4.1.2 with the exception of the glutamate concentration which was varied, whilst the specific radioactivity was kept constant. Seven different glutamate concentrations were used, ranging from 0.5 mM to 50 mM, whilst the PLP concentration was kept constant at 1 mM (see Figure 4.6).

From the data shown in Figure 4.6, the apparent $K_m$ for glutamate of chick brain GAD was calculated to be $3.7 \pm 0.4$ mM. The best straight line was calculated by the nonparametric method of Nimmo and Atkins (1979).

### 4.3.2 Apparent $K_m$ for PLP

These experiments were carried out in parallel with those described in 4.3.1. Chick brain cytosol, prepared using standard buffer from which PLP had been omitted, was desalted by gel filtration on Sephadex G25 (Section 3.1.1.2) to remove exogenous PLP and any PLP which was not very tightly associated with protein in the supernatant. The assay conditions were as described in Section 4.1.2 with the exception of the PLP concentration which was varied from 0.1 μM to 1 mM. In addition assays were performed in the absence of any added PLP. The glutamate concentration was kept constant at 25 mM.

Table 4.1 shows the specific activity (expressed as nmol GABA produced/mg protein/hr) of GAD in chick brain supernatant in the absence of PLP or in the presence of PLP at the concentrations shown. An analysis of variance showed that there was no significant difference between the values obtained for specific activity in the absence of added PLP or when PLP was added to the concentrations shown in the table.

Since GAD activity remained at the same level irrespective of whether PLP was added to the assay, it can be concluded that, using the experimental procedure described here, the enzyme remained saturated with PLP. Consequently it was not possible to determine an apparent $K_m$ for PLP.

Figure 4.6. Hanes plot for determination of $K_m$ for glutamate. See Section 4.3.1 for experimental details.

Intercept on x axis $=-K_m$. [S] = glutamate concentration (mM), v = initial reaction rate. Units = nmol GABA produced/mg protein/hr. Each point is the mean $\pm$ S.E.M., n = 3. The best straight line was calculated using the nonparametric method of Nimmo and Atkins (1979).

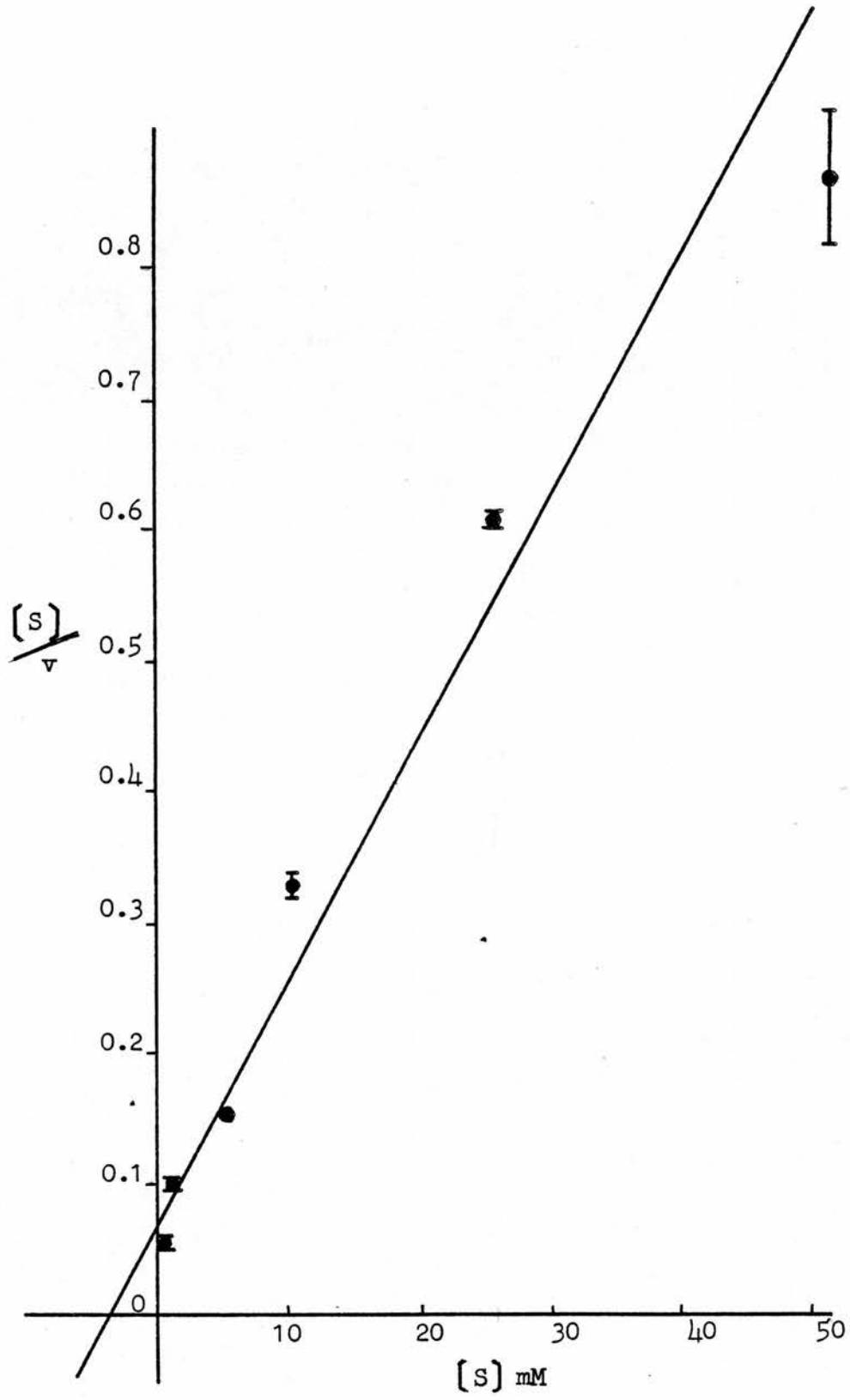

0.8
0.7
0.6
[S]/v
0.5
0.4
0.3
0.2
0.1
0
10
20
30
40
50
[S] mM

Table 4.1. The effect of varying PLP concentration on GAD activity

| PLP concentration mM | Specific activity (units*) | Fold increase over 0 PLP |
|---|---|---|
| 0 | 238 | - |
| $10^{-4}$ | 244 | 1.03 |
| $10^{-3}$ | 252 | 1.06 |
| $5x10^{-3}$ | 266 | 1.12 |
| $2.5x10^{-2}$ | 256 | 1.08 |
| $10^{-1}$ | 242 | 1.02 |
| 1 | 248 | 1.04 |

*1 unit specific activity = 1 nmol GABA produced/mg protein/hour.

The specific activity of GAD in chick brain cytosol in the presence or absence of PLP. The data is taken from 2 experiments with triplicate determinations for each concentration. An analysis of variance gave a variance ratio (F) = 0.059 for df 1 = 6 and df 2 = 6, $p \geq 0.05$. See Section 4.3.2.

## 4.4 Discussion

The assay used in this study to measure GAD activity was a modification of the radiometric assay described by Kanazawa et al (1976) in which the product of the reaction, GABA, was separated from the substrate by ion exchange chromatography. Separation of the radiolabelled product of the reaction was chosen since it was considered to be the most accurate as well as the simplest and most reproducible method of measuring GAD activity. It was confirmed that GABA was the only detectable radiolabelled product when partially purified L-[U-$^{14}$C] glutamate was used to assay GAD activity in chick brain supernatant.

The method which has been most widely used as an alternative for assaying GAD activity relies on the measurement of carbon dioxide formed during the reaction, either radiometrically (Albers & Brady 1959; Roberts & Simonsen 1963) or using a Warburg apparatus (Roberts & Frankel 1951). However, there have been a number of reports of discrepancies when values obtained for GAD activity by measuring the amount of radiolabelled carbon dioxide liberated were compared with those obtained by measuring the amount of radiolabelled GABA formed (Wilson et al 1972; Kanazawa et al 1976). Both authors concluded that alternative metabolic pathways were contributing to the production of carbon dioxide. Further experiments by Kanazawa et al (1976) indicated that any alternative metabolic pathways which might be operating did not affect the production of radiolabelled GABA in the assay.

The presence of contaminants in commercial [1-$^{14}$C] glutamate which release radiolabelled carbon dioxide during incubation have been reported (Miller & Martin 1973). The possibility that carbon dioxide might be released by decarboxylation of pyroglutamic acid, identified as a contaminant of radiolabelled glutamic acid (Drummond & Phillips 1974), following acidification was suggested (Gonnard & Whicker 1974).

Immunochemical studies have indicated that GAD from avian brain differs greatly from GAD from mouse, rat, human or calf brain (Saito et al 1974). Since the assay conditions used to determine GAD activity had been developed using the mammalian enzymes, experiments were carried out to characterise the GAD assay for avian GAD before it was used on a routine basis. The extent to which GABA production was linear with

respect to time and protein concentration were established and the effect of varying the pH and the glutamate and PLP concentrations of the assay were investigated.

Human GAD activity is optimal at pH 6.8 (Blindermann et al 1978(a)) and both mouse and rat GAD activities are optimal at pH 7 (Wu et al 1973; Maitre et al 1978). Chick brain GAD activity is optimal at about pH 7 and therefore does not differ greatly in this respect. This suggests the possibility that although structurally avian and mammalian GAD may differ markedly, the environment of the active site may be similar. There is evidence to suggest that GAD is not highly conserved during evolution (Saito et al 1974). Using immunochemical methods it was demonstrated that whereas GAD was similar in several mammalian species, frog, avian and fish GAD were very different.

An apparent $K_m$ for glutamate for chick brain GAD was calculated to be 3.7 mM. This compares with 0.7 mM, 1.1 mM, 1.28 mM and 1.6 mM for mouse (Wu et al 1973), rat (Maitre et al 1978), human (Blindermann et al 1978(a)) and bovine (Wu 1982) brain GAD respectively. Although the $K_m$ value determined for chick brain GAD is slightly higher than those calculated for the other species, this may just be the result of generic differences between birds and mammals. The differences in $K_m$ for glutamate of GAD from the different species may reflect the structural differences demonstrated immunochemically (Saito et al 1974).

Preliminary experiments to determine an apparent $K_m$ for PLP indicated that GAD present in a chick brain supernatant remained saturated with PLP, even after desalting on Sephadex G25. This implies that the association between enzyme and cofactor is very tight. This is consistent with evidence that, in the absence of glutamate, PLP is very tightly bound to GAD (Miller et al 1978). Further experiments involving the association of PLP with GAD are described in Section 7. Difficulties involved in trying to determine a $K_m$ for PLP for GAD and any evidence for a role for PLP in the control of enzyme activity will be discussed in Section 7.

A $K_m$ for glutamate for chick brain GAD will only be determined unequivocably using a pure protein preparation which will ensure there are no unknown factors modifying enzyme activity and hence affecting the $K_m$. As a major inhibitory neurotransmitter in the CNS GABA has an important role in determining the overall level of neuronal

excitability. Consequently there must be a mechanism by which GABA levels in the brain are controlled. At present the mechanism by which GABA synthesis is regulated has not been elucidated consequently little is known about factors which may be involved in controlling or modulating GAD activity. It seems unlikely, in view of the high concentration of glutamate throughout the brain, that control of enzyme activity will be effected by substrate availability. There is no evidence either for or against the presence of an "effector" or "modulator" molecule which regulates GAD activity.

Any evidence for possible mechanisms regulating GAD activity and, therefore, GABA synthesis will be discussed further in Section 7.

## SECTION 5: PURIFICATION OF CHICK BRAIN GAD

### 5.1 Purification protocol

#### Tissue preparation

The brains from 70 to 140 broilers, 6 - 12 months old, (Marshalls Ltd, Newbridge, Midlothian) were homogenised 1:10 (w/v) in standard buffer containing 0.4 mM PMSF as a serine protease inhibitor (Fahrney & Gold 1963) using an Ystral tissue macerater. The homogenate was centrifuged for 1 hour at 100 000 $g_{av}$, 4°C, and the resulting supernatants pooled. The total volume of the pooled supernatant was between 1100 ml and 2500 ml. A sample of approximately 250 ml was removed for immediate processing and the remainder was made 20% (v/v) with respect to glycerol and stored at -20°C until use.

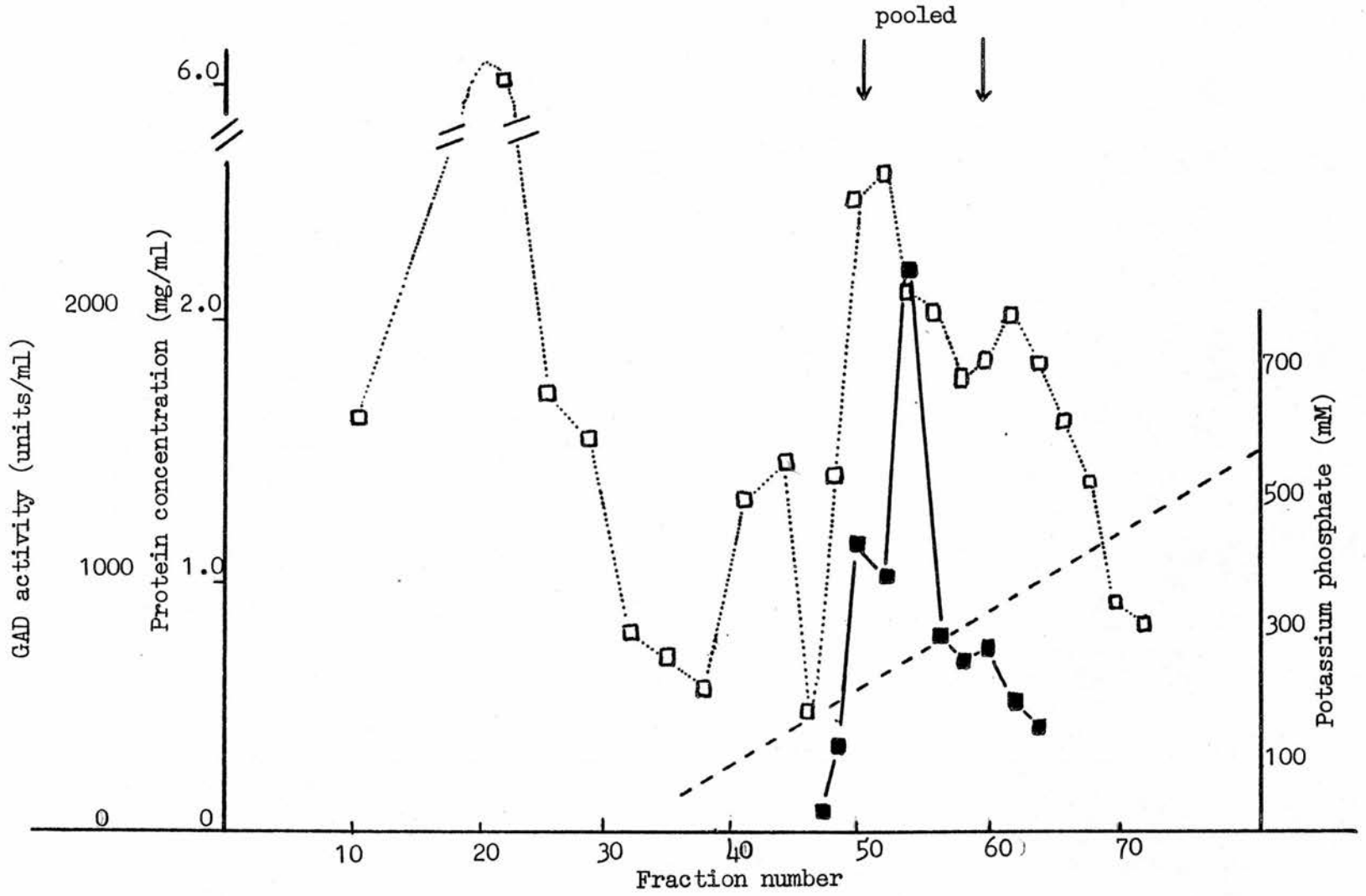
Batches of the supernatant (between 100 and 150 ml) were desalted and the buffer concentration changed by gel filtration on a column of Sephadex G25 (5.4 cm x 27 cm) as described in Section 3.1.1.2. This replaced lengthy dialyses and reduced the amount of time required for the purification which minimised losses of GAD activity.

#### Chromatography on DEAE cellulose

After desalting by gel filtration on Sephadex G25 and the addition of PMSF (final concentration 0.4 mM) up to 300 ml of the supernatant was applied (100 ml/hour) to a column (2.5 cm x 42.5 cm) of DEAE cellulose which had previously been equilibrated in standard buffer at 4°C. After washing with 1 bed volume of standard buffer (100 ml/hour) the proteins were eluted with a linear gradient of 800 ml potassium phosphate (50 mM to 750 mM) containing 0.1 mM PLP and 1 mM AET, pH 7.4. The elution rate was approximately 50 ml/hour. The first 100 ml of the wash was discarded then 13.6 ml fractions were collected. On a number of occasions these fractions were assayed for phosphate (using the method described in Section 3.6.3) to check the gradient. Those fractions containing the highest GAD activity (assayed as described in Section 4.1.2) were pooled and desalted by gel filtration on Sephadex G25 into 5 mM potassium phosphate buffer; pH 7.4, containing 0.1 mM PLP and 1 mM AET. Protein concentration was determined by the method of Lowry *et al* (1951). Figure 5.1 shows the elution profile obtained.

Figure 5.1. Chromatography on DEAE cellulose of chick brain supernatant. See Section 5.1.

Fraction volume, 13.6 ml. The fractions containing the highest GAD activity, indicated by arrows, were pooled. One unit of enzyme activity = 1 nmol GABA produced/hour. □ - □, protein concentration; ■ - ■, GAD activity.


pooled
6.0
2000
2.0
1000
1.0
0
0
GAD activity (units/ml)
Protein concentration (mg/ml)
700
500
300
100
Potassium phosphate (mM)
10
20
30
40
50
60
70
Fraction number

Chromatography on hydroxyapatite

The synthesis of the hydroxyapatite and preparation of the column are described in Section 3.1.3. PMSF was added (final concentration 0.4 mM) to the desalted peak fractions collected from the DEAE cellulose column. These were then adsorbed (40 ml/hour) on to a hydroxyapatite column (2 cm x 8 cm) previously equilibrated with 5 mM potassium phosphate buffer. Proteins were eluted with a series of steps of increasing potassium phosphate concentration, pH 7.4, containing 0.1 mM PLP and 1 mM AET, as follows; 25 ml 5 mM potassium phosphate (40 ml/hour) followed by 25 ml 50 mM potassium phosphate, 25 ml 150 mM potassium phosphate and 25 ml 300 mM potassium phosphate all at 80 ml/hour. Figure 5.2 shows the elution profile obtained. Those fractions containing the highest enzyme activity were retained for chromatography on Sephadex G200. Protein concentration was determined by the method of Lowry et al (1951).

Chromatography on Sephadex G200

For calibration of the Sephadex G200 column see Section 3.1.1.2. If the volume of the active fraction obtained from hydroxyapatite chromatography exceeded 10 ml, the sample was concentrated using dry Sephadex G25 as described in Section 3.3.2. Samples of less than 10 ml were applied directly to a column (1.8 cm x 95 cm) of Sephadex G200 previously equilibrated with standard buffer. The proteins were eluted with standard buffer at a rate of 10 ml/hour. The elution profile is shown in Figure 5.3. Protein concentration was determined by the method of Lowry et al (1951). The peak fractions were pooled and stored in 20% (v/v) glycerol at $-20^{o}C$.

Table 5.1 shows the degree of purification of chick brain GAD achieved using this procedure. Compared with the supernatant the enzyme was purified 25 fold with an activity yield of 3.1%. The protein concentration was decreased about 800 fold.

At each stage of the procedure samples were removed and analysed by non denaturing polyacrylamide gel electrophoresis and electrophoresis on 11% and 15% (acrylamide w/v) denaturing SDS/urea slab gels to assess the progress of the purification, Figures 5.4a and b, 5.5 and 5.6 respectively. Details of the preparation of the gels, conditions of electrophoresis and staining of proteins in the gels are described in Section 3.2.

Figure 5.2. Chromatography on hydroxyapatite of the pooled peak fractions from the DEAE cellulose column. See Section 5.1.

Volume of fractions 1-17 inclusive, 9 ml; volume of fraction 18 onwards, 5.6 ml. Those fractions containing the highest GAD activity (25-27 inclusive) were pooled. One unit of enzyme activity = 1 nmol GABA produced/hour. (———) indicates the potassium phosphate concentrations used for elution. □ - □ , protein concentration; ■ - ■ , GAD activity.

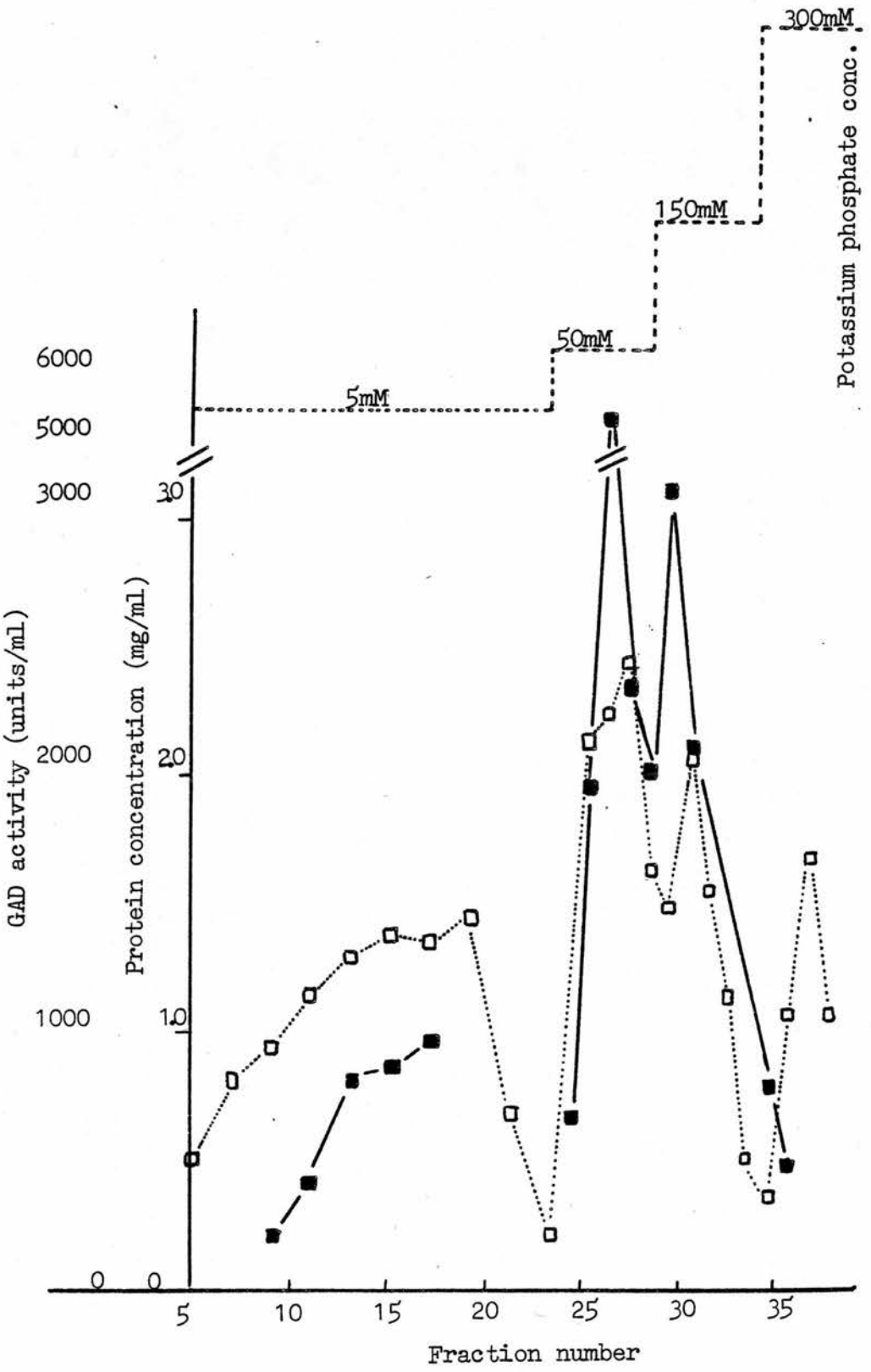

300mM
150mM
50mM
5mM
Potassium phosphate conc.
6000
5000
3000
2000
1000
0
30
20
10
0
GAD activity (units/ml)
Protein concentration (mg/ml)
5
10
15
20
25
30
35
Fraction number

Figure 5.3. Chromatography on Sephadex G200 of the pooled fractions from the hydroxyapatite column. See Section 5.1.

Fraction volume, 1.8 ml. The fractions containing the highest GAD activity, indicated by arrows, were pooled. One unit of enzyme activity = 1 nmol GABA produced/hour. □-□ , protein concentration; ■-■ , GAD activity.

GAD activity (units/ml)
Protein concentration (mg/ml)
Fraction Number
pooled
1000
500
0
1.0
0.5
0
30
40
50
60
70
80
90
100

Table 5.1. Purification of chick brain GAD

| Fraction | Total Protein (mg) | Specific Activity (units/mg protein) | Purification (-fold) | Total Activity (units) | Activity Yield (%) | |
|---|---|---|---|---|---|---|
| Supernatant (post G25) | 2123 | 0.231 | - | 491 | - | |
| DEAE cellulose peak fractions (post G25) | 273.6 | 0.480 | 2.1 | 131 | 27 | |
| Hydroxy-apatite | | | | | | |
| *peak 1 | 33.3 | 1.291 | 5.6 | 43 | 8.8 | 15.3 |
| peak 2 | 28.6 | 1.119 | 4.8 | 32 | 6.5 | |
| Sephadex G200 peaks | 2.71 | 5.667 | 24.5 | 15.4 | 3.1 | |

1 unit of GAD activity = 1 umol GABA produced/hour.

*Peak 1 applied to Sephadex G200.

The purification of chick brain GAD is described in Section 5.1.

Non denaturing polyacrylamide gel electrophoresis of the peak fractions from the Sephadex G200 column revealed 3 bands, 1 strong and 2 much weaker (Figures 5.4a and b). When denaturing SDS/urea polyacrylamide gel electrophoresis was carried out multiple bands could be distinguished on both the 11% and 15% gels (Figures 5.5 and 5.6).

## 5.2 Elution of proteins from non denaturing 'activity' gels

### 5.2.1 Elution into standard buffer

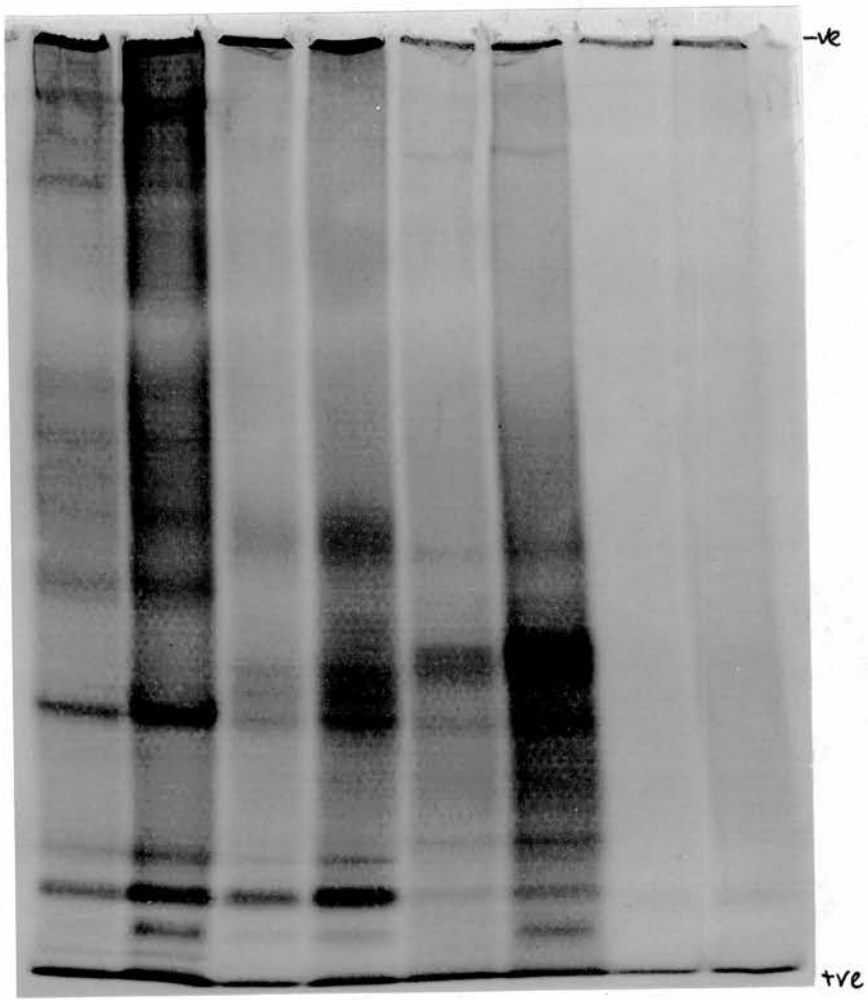
A chick brain supernatant was prepared by centrifuging an homogenate, 1:10 (w/v) in standard buffer, at 100 000 $g_{av}$, 4°C, for 1 hour. The supernatant was concentrated as described in Section 3.2.2 and 50 ul samples (containing 500 µg protein) loaded onto 5% (w/v acrylamide) "activity" disc gels followed by electrophoresis as described in Section 3.2.1.2.1.

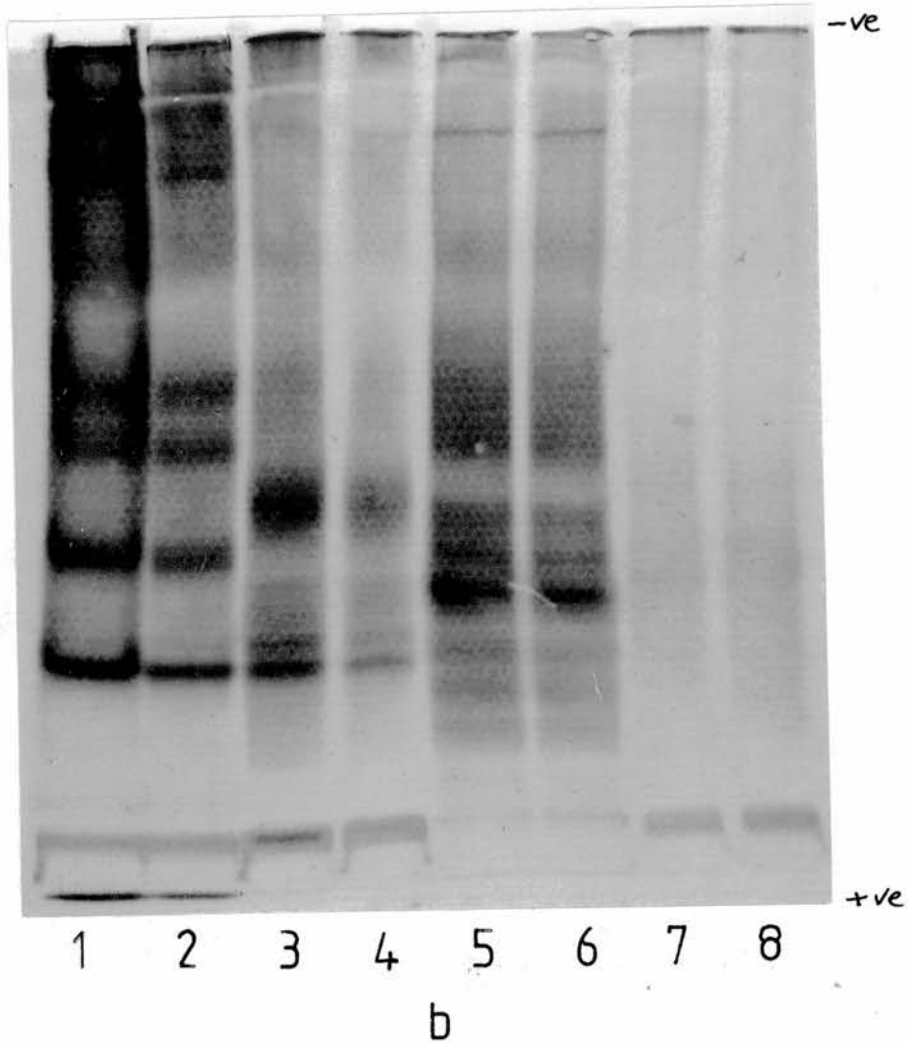
Figure 5.7 shows the results obtained by pooling gel segments from 7 disc gels with elution periods of either 4 hours or overnight (approximately 15 hours). An increase in the elution period did not greatly alter the recovery of the GAD activity which appeared to be located in a diffuse band over the top 20%-25% of the gel. A total of 2317 units of GAD activity (1 unit is 1 nmol GABA produced/hour) were loaded on to the 7 gels. After 4 hours 15.7% of the activity was recovered in the elution buffer and after 15 hours 18.2% was recovered. Since electrophoresis may cause some loss of enzyme activity, as much as 50% of the activity of catfish brain GAD was lost due to electrophoresis (Su et al 1979), then recovery by elution may be more efficient than it appears.

Alternatively, it is possible that these values for recovery of enzyme activity may have been overestimated. They were obtained by addition of the results obtained from GAD assays performed on the eluted proteins from consecutive regions of the gels. The number of dpm obtained per assay was low, the highest being approximately 500 dpm, therefore the errors inherent in the calculation of dpm in liquid scintillation counting will comprise a much larger percentage of the final value. Addition of the values obtained for the eluted proteins from more than one region of the gel to give a total value for the recovery will magnify any errors resulting in an overestimation of the percentage recovery of activity.

Figure 5.4.

Non denaturing polyacrylamide gel electrophoresis, 5% acrylamide (w/v). Tracks 1 and 2, supernatant, 125 μg and 250 μg protein respectively; Tracks 3 and 4, peak fractions from DEAE cellulose column, 40 μg and 90 μg protein respectively; Tracks 5 and 6, peak fractions from hydroxyapatite column, 47 and 94 μg protein respectively; Tracks 7 and 8, peak fractions from Sephadex G200 column, 20 μg and 40 μg protein respectively. (a) Gel stained with Coomassie Brilliant Blue (Section 3.2.3.1). (b) Gel stained using the silver staining method of Wray et al (1982) (Section 3.2.3.2). Conditions of electrophoresis are described in Section 3.2.1.


-ve
+ve
1 2 3 4 5 6 7 8
a


-ve
+ve
1 2 3 4 5 6 7 8
b

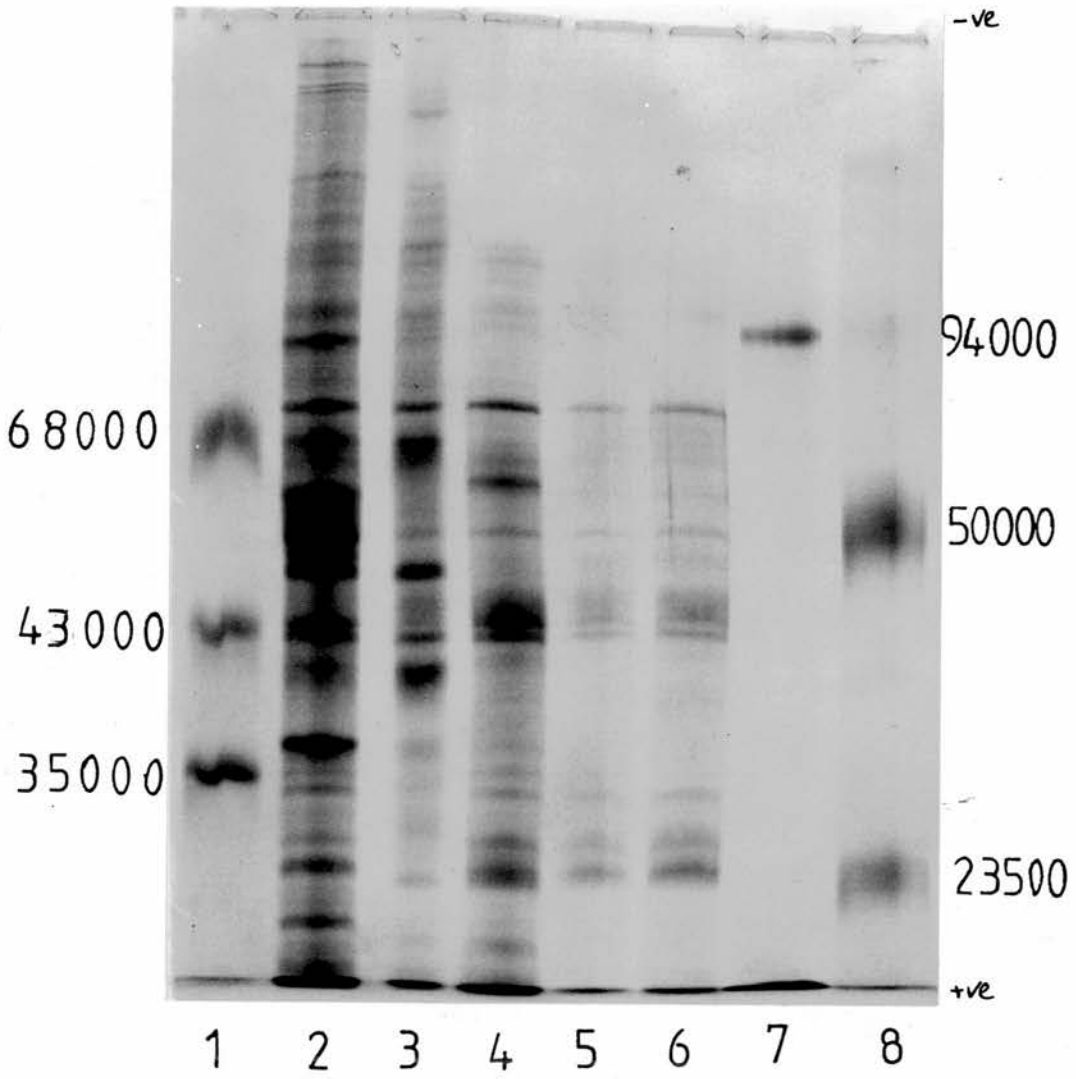



Figure 5.5 SDS/urea denaturing polyacrylamide gel electrophoresis (11% acrylamide, W/v). Tracks 1,7 and 8, $M_r$ marker proteins; Track 2, supernatant, 125 µg protein; Track 3, peak fractions from DEAE cellulose column, 45 µg protein; Track 4, peak fractions from hydroxyapatite column, 47 µg protein; Tracks 5 and 6, peak fractions from Sephadex G200 column, 10 µg and 20 µg protein respectively. Conditions of electrophoresis are described in Section 3.2.2.

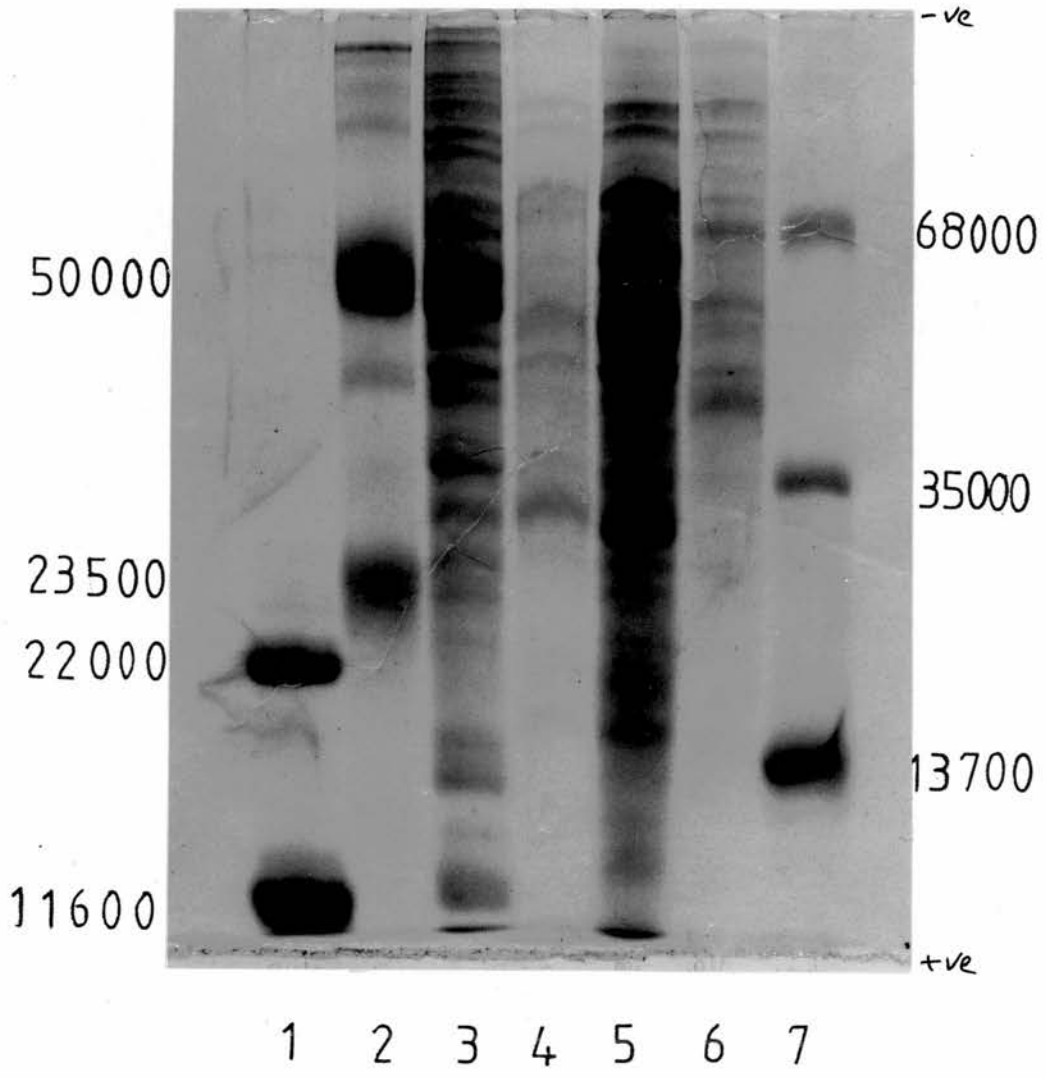



Figure 5.6 SDS/urea denaturing polyacrylamide gel electrophoresis (15% acrylamide $^{w}/_{v}$). Tracks 1,2 and 7, $M_r$ marker proteins; Track 3, supernatant, 125 μg protein; Track 4, peak fractions from Sephadex G200 column, 20 μg protein; Track 5, peak fractions from DEAE cellulose column, 45 μg protein; Track 6, peak fractions from hydroxyapatite column, 47 μg protein. Conditions of electrophoresis are described in Section 3.2.2.

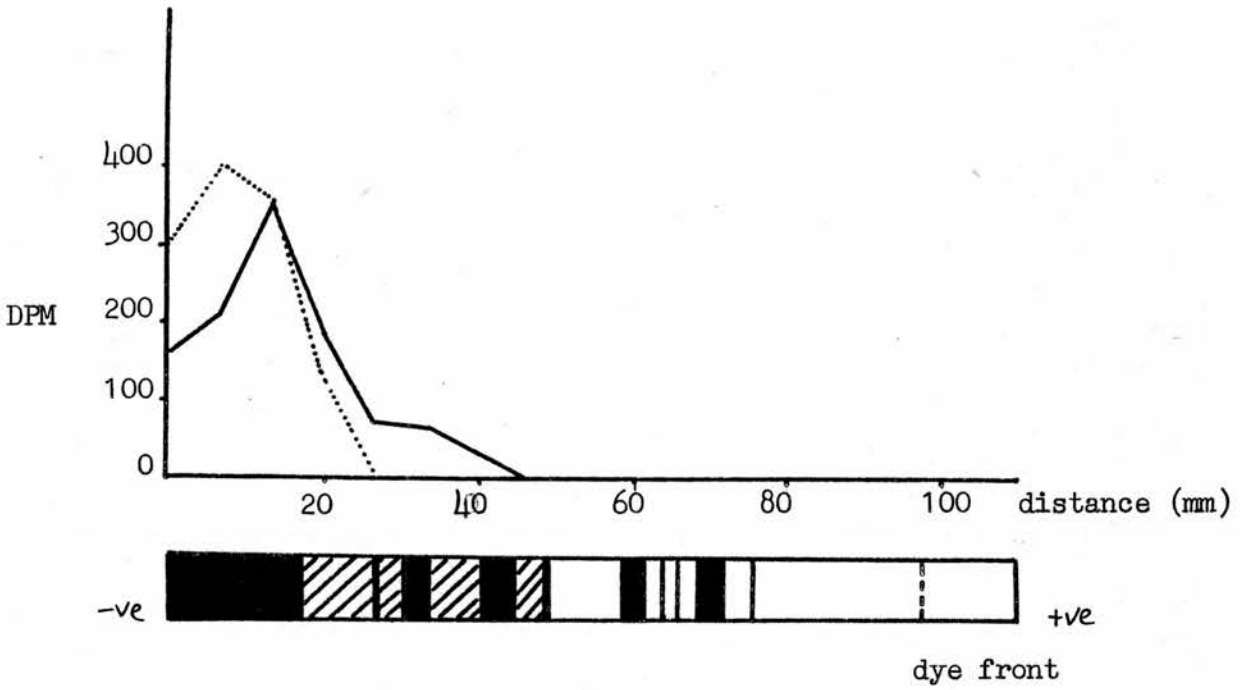



Figure 5.7 A diagrammatic representation of the protein pattern obtained after polyacrylamide gel electrophoresis of chick brain supernatant on a 5% acrylamide, w/v, 'activity' disc gel. The GAD activity eluted from seven parallel disc gels is expressed as d.p.m. / pooled gel slices (6mm). (—) elution for 4 hours, (.....) elution overnight. See section 5.2 for details.

Increasing the volume of the elution buffer would improve the efficiency of diffusion of the protein out of the gel but the resulting dilution would make it impossible to detect GAD activity. It was not possible to increase the amount of protein loaded per gel and, therefore, the amount of GAD activity per gel since the gels had already been overloaded and this would result in even more reduced resolution.

When the experiment was repeated using partially purified samples containing GAD activity, for example the peak fractions obtained from chromatography on DEAE cellulose or hydroxyapatite it proved impossible to detect any GAD activity in the eluted proteins.

5.2.2 Elution by electrophoresis

The sample was prepared as described in Section 5.2.1 and a total volume of 800 ul (between 6 mg and 8 mg of protein) loaded on to a 5% "activity" slab gel followed by electrophoresis as described in Section 3.2.1.1. The proteins were eluted from slices of gel as described in Section 3.2.1.2.2.

Although protein was eluted from the gel segments and concentrated at the bottom of the dialysis bag, GAD assays which were performed on the eluted proteins failed to detect any GAD activity.

5.3 Determination of an approximate $M_r$ for chick brain GAD

The approximate $M_r$ for chick brain GAD was determined by gel filtration on Sephadex G200 as described in Section 3.1.1.1. Using this method an $M_r = 150\ 000 \pm 15\ 000$ (n = 4) was calculated.

## 5.4 Discussion

GAD from several vertebrate species has been purified (Wu et al 1973; Hadjian & Stewart 1977; Blindermann et al 1978(a); Maitre et al 1978; Su et al 1979; Wu 1982) using chromatographic techniques similar to those used for chick brain GAD. Ammonium sulphate fractionation was included in the purification of mouse, catfish and bovine brain GAD (Wu et al 1973; Su et al 1979; Wu 1982) to precipitate much unwanted protein. However, ammonium sulphate fractionation of chick brain supernatant resulted in substantial losses of GAD activity. This could be due to the presence of trace contaminants in the ammonium sulphate which were sufficient to affect the enzyme activity. However, ammonium sulphate fractionation caused large losses of rat and human brain GAD activity (Blindermann et al 1978(b)) indicating that ammonium sulphate fractionation may be responsible for the activity loss. Since 75% saturation with ammonium sulphate results in a concentration of 4M ammonium sulphate, it is possible that the high ionic concentration may cause denaturation. Alternatively, interaction between sulphate ions and positively charged amino acids on the surface of the protein may cause charge shifts sufficient to result in denaturation. Since there is evidence to suggest that the active site of GAD may be positively charged (Wu & Roberts 1974; Wu 1976), sulphate ions may interact with positively charged amino acids at the active site resulting in inactivation. It is interesting that sodium sulphate fractionation has also been reported to cause loss of mouse brain GAD activity (Susz et al 1966).

The protocol finally adopted to try to isolate chick brain GAD resulted in a 25 fold purification with respect to the supernatant, see Table 5.1. This was accompanied by an 800 fold reduction in protein concentration and was represented by 3 bands on a non denaturing polyacrylamide gel (Figure 5.4). These facts suggest that GAD protein may have been enriched more than the data indicate and that the value for "-fold purification" has been artefactually lowered due to denaturation and loss of enzyme activity. Further column chromatography after Sephadex G200 proved impossible since enzyme activity was reduced to undetectable levels. Storage of fractions from Sephadex G200 columns in 20% (v/v) glycerol at $-20^{\circ}C$ so that the products of large

amounts of material could be pooled for future processing also proved to be of limited value since, despite the protective effect of glycerol (Blindermann et al 1978(b)) and careful concentration of protein, enzyme activity was lost.

It is interesting to note that a minor low molecular weight peak of GAD activity was detected after gel filtration on Sephadex G200. There has been a report of a minor high molecular weight peak of GAD activity which was excluded from the gel during chromatography with Sephadex G200 (Wu et al 1976). This proved to be immunologically and kinetically the same as the major peak. However, other groups have not detected the high molecular weight species (Blindermann et al 1978(a)). There have been no reports of a minor low molecular weight species.

The lower molecular weight species observed here may be artefactual, for example, the product of partial proteolysis. Alternatively it may represent a low molecular weight form of the enzyme which constitutes a small percentage of the total enzyme activity. It is possible that for full activation GAD may have to associate with another, as yet unidentified, molecule converting it to the major higher molecular form.

Protease activity probably contributed to the loss of enzyme activity during the early stages of the purification although PMSF was added at several stages to try to prevent the action of serine proteases, the most active at the pH of the buffers used (Barrett 1977). However, chick brain GAD appears to be an unstable enzyme, despite the presence of PLP and AET in all the buffers, particularly when the protein concentration is reduced. The addition of PLP (Roberts & Simonsen 1963; Susz et al 1966) and AET (Susz et al 1966) has been shown to stabilise GAD activity. AET will maintain any sulphydryl groups in a reduced state. There is evidence that there may be at least one sensitive sulphydryl group, at or near the active site, which is required for activity (Wu & Roberts 1974).

Greater success has been achieved in the purification of GAD from other species. Human brain GAD has been purified 8 000 fold (Blindermann et al 1978(a)) and rat brain GAD about 3 000 fold (Maitre et al 1978) over the original homogenate. In each case the enzyme was judged to be pure by a number of criteria including the fact that the protein was homogeneous with respect to mobility on both denaturing and non denaturing polyacrylamide gels and, in the latter case, all the

enzyme activity was associated with the one band. Since the molecular weight obtained for pure human and rat brain GAD under denaturing conditions was approximately half that calculated for the undenatured enzyme, and only one N-terminal amino acid was detected, it was concluded that the enzyme was a dimer with two identical subunits (Blindermann et al 1978(a); Maitre et al 1978).

Mouse brain GAD has been purified 700 fold over the supernatant. Non denaturing polyacrylamide gel electrophoresis revealed one major protein band with which all the enzyme activity was associated and one other faint band (Wu et al 1973). However, when this preparation was analysed by polyacrylamide gel electrophoresis under denaturing conditions multiple bands were revealed with $M_r$s ranging from 15 000 to 129 000. Four major bands had $M_r$s ranging from 60 000 to 120 000 (Matsuda et al 1973). This was interpreted as being due to dissociation and association, 15 000 being the smallest dissociable subunit, and that six of these small polypeptides might be associated together to form two larger identical subunits. Since comparison of immunochemical data (Saito et al 1974; Maitre et al 1978) suggests that there is no significant difference between mouse, rat and human brain GAD, these discrepancies in electrophoretic pattern have been explained as due to differences in methodology (Sze 1978) or the presence of contaminants (Blindermann et al 1978(b)).

An alternative explanation could be that the low molecular weight bands were the result of proteolysis by a protease which had copurified with mouse brain GAD but, due to the different protocols employed, was absent in the rat and human preparations. Incubation at 37°C or 60°C with SDS will permit the retention of proteolytic activity (Gordon 1975). The high molecular weight bands could be due to incomplete denaturation of the protein since, unless the protein is incubated at a temperature and for a period of time which would normally cause denaturation, the addition of SDS will not necessarily result in complete denaturation (Gordon 1975). Alternatively there may have been some reaggregation of the polypeptides. The addition of urea eliminates this problem (Swank and Munkres 1971), however, dialysis of the samples prior to electrophoresis to remove the urea may also cause the dissociation of a small amount of SDS from the protein (Shapiro & Maizel 1969), sufficient to allow limited reaggregation.

There is evidence of proteolytic activity from the multiple bands obtained on the SDS/urea polyacrylamide gels shown in Figures 5.5 and 5.6. Since the sample contained more than one protein, multiple bands under denaturing conditions would be expected but there are at least 10 polypeptides, for which the molecular weight has been estimated, plus a few very faint bands which can barely be distinguished. Although the samples were heated rapidly to greater than 60°C there could still have been opportunity for limited proteolysis. It is unlikely that reaggregation occurred since urea was present in both the samples and polyacrylamide gel. Any proteolytic fragments generated during the purification of GAD would have been removed by chromatography on Sephadex G200 unless the protein had contained disulphide bonds and hence retained structural integrity until SDS polyacrylamide gel electrophoresis. There is evidence that mammalian GAD does not contain disulphide bonds (Maitre et al 1978) however, care must be employed when relating properties shown for mammalian GAD to avian GAD since the two enzymes have been shown, immunologically, to be very different (Saito et al 1974).

It was hoped that preparative polyacrylamide gel electrophoresis of partially purified GAD which had retained a reasonably high level of enzyme activity would solve the problem of enzyme instability and yield pure GAD. Preparative gel electrophoresis had been used with some success in the purification of bovine brain GAD (Hadjian & Stewart 1977; Wu 1982) and catfish brain GAD (Su et al 1979). However, the pilot experiments indicated that enzyme instability might again cause problems, mainly that of loading sufficient enzyme activity to be detectable after electrophoresis and elution. The application of very concentrated samples of a supernatant resulted in the elution of a small proportion of the activity loaded (Figure 5.7). This was however, located at the top of the gels and did not coincide with any of the three bands present in the most purified sample (Figure 5.4). As can be seen from the gels in Figure 5.4, in each track a large proportion of the protein loaded has remained at the top of the gel. It is possible that most of the GAD in the protein sample applied remained at the top of the gel so that the GAD which was separated by electrophoresis was not present in sufficient amounts to be detected after elution: GAD activity could not be detected after electrophoresis of partially purified GAD probably due

to a combination of being unable to load sufficnt GAD in relation to the total protein concentration and the enzyme's instability to the conditions of electrophoresis.

Samples of concentrated supernatant were electrophoresed on slab gels since as much as 8 mg protein could be applied. However, although protein could be successfully eluted by electrophoresis, GAD activity could not be. This may be attributed to the increased length of time GAD was exposed to the electrophoretic conditions which caused increased loss of activity. Since it was not possible to identify which of the three proteins separated by non denaturing polyacrylamide gel electrophoresis corresponded to GAD, molecular weight analysis using SDS polyacrylamide gel electrophoresis could not be carried out.

A tentative $M_r$ of 150 000 $\pm$ 15 000 was assigned to chick brain GAD by gel filtration on Sephadex G200. This compares with $M_r$ = 140 000 for human and rat brain GAD (Blindermann et al 1978(a); Maitre et al 1978) and $M_r$ = 90 000 for mouse and catfish brain GAD (Wu et al 1973; Su et al 1979). The $M_r$ calculated for chick brain GAD is an approximate value. Any $M_r$ determined by gel filtration, without confirmation using other techniques can only be regarded as tentative since the calculation relies on assuming that the protein is completely spherical. In addition, the possibility of interaction between the protein and the gel matrix or of aggregation cannot be discounted.

It did not prove possible to purify chick brain GAD to homogeneity using conventional techniques. Successful purification would necessitate that the partially purified extracts from very large quantities of tissue could be pooled for future processing. Unfortunately chick brain GAD lost activity so rapidly that it was not possible to detect activity in the peak fractions from the Sephadex G200 column after storage at -20°C.

## SECTION 6: AFFINITY CHROMATOGRAPHY

### 6.1 Methods

#### 6.1.1 Preparation of the gel for the PMP-Sepharose 4B column

The PMP-Sepharose 4B affinity gel was synthesised as described by March et al (1974)

Cyanogen bromide activation of Sepharose 4B

One volume (25 ml) washed Sepharose 4B slurry (equal volumes of gel and double distilled water) was added to 1 volume 2M sodium carbonate and stirred slowly. The rate of stirring was increased and 0.05 volumes of acetonitrile containing cyanogen bromide, 2 g cyanogen bromide/1 ml acetonitrite was added. After vigorous stirring for 1-2 minutes the slurry was washed, as rapidly as possible, on a coarse scintered glass funnel, with between 5 and 10 volumes ice cold 0.1M $NaHCO_3$, pH 9.5. This was followed with between 5 and 10 volumes of double distilled water and finally 0.1M $NaHCO_3$, pH 9.5 which was the buffer used in the coupling reaction. After the final wash the slurry was filtered under vacuum to produce a moist cake.

Coupling reaction

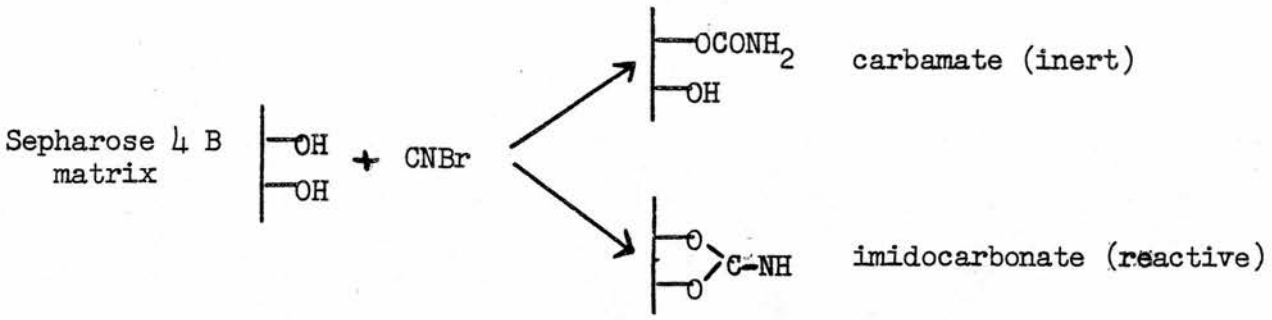
The moist cake of activated Sepharose 4B was placed in a plastic bottle containing 1 volume (25 ml) 0.1M $NaHCO_3$, pH 9.3 and 6 g diaminohexane. This was mixed then left for 18-20 hours at $4^{o}C$ on an end-over-end shaker for the coupling reaction to occur.

Addition of PLP

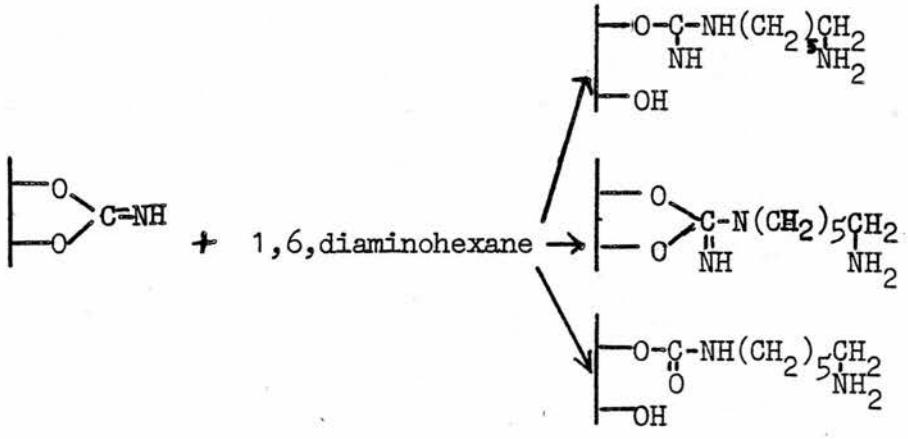
After 18-20 hours the gel was washed, on a coarse sintered glass funnel, under vacuum with 1.5 1 double distilled water. The moist cake of gel was added to 1 volume (25 ml) double distilled water and 0.1 g PLP added. The slurry was stirred gently for 30 minutes. The beaker containing the slurry was transferred to ice and 40 mg sodium borohydride was added slowly with gentle stirring throughout. The slurry was left to stir until no more hydrogen was evolved and then washed with 1 1 ice cold double distilled water. The PLP coupling step was repeated twice more. After the final wash with distilled water the slurry was washed with 2M KCl. The aminohexyl pyridoxamine phosphate Sepharose 4B (PMP-Sepharose 4B) was stored in 1M KCl at $4^{o}C$ until use.

Figure 6.1. Schematic diagram showing the steps involved in coupling PLP to Sepharose 4B. Method of preparation is described in Section 6.1.1.

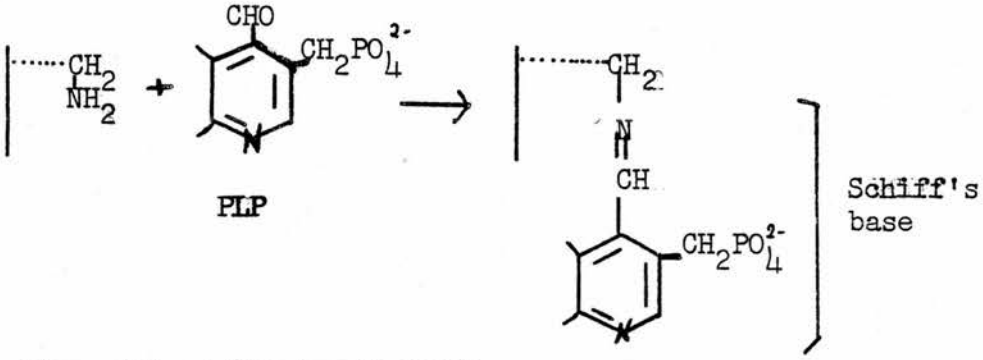
Cyanogen bromide activation.



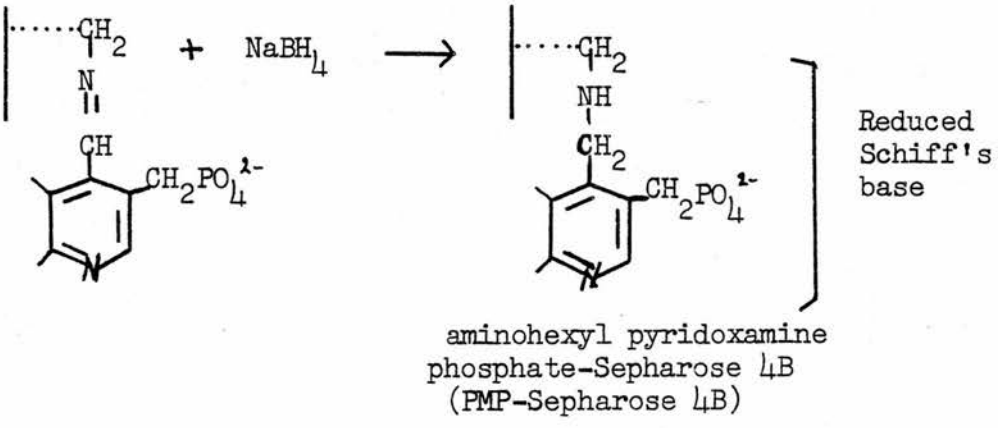

Coupling of diaminohexane




Addition of PLP




Reduction with sodium borohydride




aminohexyl pyridoxamine phosphate-Sepharose 4B (PMP-Sepharose 4B)

6.1.2 Preparation of aminohexyl pyridoxamine phosphate

Melted diaminohexane (28 µl) was added to 50 ml 0.1M $NaHCO_3$, pH 9.5, containing 25 mg PLP and left to stir at room temperature for 20 minutes. The solution was then cooled on ice (to below 10°C) and 40 mg sodium borohydride added slowly, stirring throughout. After the addition of sodium borohydride was complete the solution was stirred for a further 10 minutes and then at room temperature until no more hydrogen was evolved. Samples of this solution were diluted in 0.1 M NaOH for examination of the absorbance spectrum and of the excitation and emission spectra.

6.1.3 Spectrofluorimetric and spectrophotometric analysis of the PMP-Sepharose 4B gel

6.1.3.1 Spectrofluorimetric analysis

Between 10 ml and 15 ml of the PMP-Sepharose 4B gel was washed with 0.1M KCl followed by distilled water. A dilute suspension of this gel was placed in a quartz cuvette and mixed thoroughly to ensure even distribution of the beads. The PMP-Sepharose 4B proved to be fluorescent when irradiated with light of the correct wavelength. The excitation and emission spectra of the gel, using a blank of untreated gel, were compared with the excitation and emission spectra of an aminohexyl pyridoxamine phosphate solution, using a blank of diaminohexane, on a Perkin-Elmer fluorescence spectrophotometer.

6.1.3.2 Spectrophotometric analysis

The washed PMP-Sepharose 4B was resuspended in 0.1M NaOH. A dilute suspension of the gel was placed in a quartz cuvette and mixed thoroughly to ensure even distribution of the beads. The absorbance spectrum of the gel, measured versus a blank of untreated gel in 0.1M NaOH on a Pye Unicam SP 1800 double beam spectrophotometer, was compared with that of aminohexyl pyridoxamine phosphate read versus a blank of diaminohexane diluted with 0.1M NaOH.

6.1.4 Chromatography on PMP-Sepharose 4B

A number of small scale pilot experiments were performed to test the efficiency of the PMP-Sepharose 4B affinity gel at binding chick brain GAD.

Two 3 week old chicks were decapitated and their brains homogenised, 1:5 (w/v), in ice cold double distilled water containing 0.1% Triton X100 (w/v) and 1 mM PMSF. The homogenate was centrifuged at 100 000 $g_{av}$ for 1 hour

and the supernatant put into the correct buffer by gel filtration on a column of Sephadex G25 which had been equilibrated with 20 mM imidazole acetate, pH 6.5, containing 1 mM AET. After a 15 minute preincubation at 37°C with 100 mM glutamate (see Section 7) the supernatant was passed through a second Sephadex G25 column which had been thoroughly washed with distilled water. The resulting sample was loaded (15 ml/hour) onto a small (5 ml packed bed volume) PMP-Sepharose 4B column, in distilled water. The column was washed with 3 ml distilled water followed by approximately 5 bed volumes 10 mM potassium phosphate, pH 7.2 then 100 mM potassium phosphate pH 7.2. Fractions (2 ml) were collected, made 500 uM with respect to PLP then, after a 10 minute interval in ice, assayed for GAD activity as described in Section 4.1.2. Protein concentration was determined by the method of Lowry et al (1951).

Alternatively, after the second gel filtration step, the sample was added to 5 ml (packed volume) of washed PMP-Sepharose 4B. This was left for 30 minutes at 4°C, stirring just quickly enough to keep the gel dispersed throughout the sample so that the protein could bind to the gel. The slurry was then poured into a glass column and protein eluted as previously described. Fractions (2 ml) were collected.

## 6.2 Results and Discussion

### 6.2.1 Spectrofluorimetric and spectrophotometric analysis of the PMP-Sepharose 4B gel

#### 6.2.1.1 Spectrofluorimetric analysis

The PMP-Sepharose 4B gel and aminohexyl pyridoxamine phosphate solution had identical excitation and emission spectra, with the excitation maximum at 310 nm and the emission maximum at 365 nm. This suggests that PLP has been linked to the Sepharose 4B.

The amount of PLP covalently bound to the Sepharose 4B was estimated by comparing the height of the peaks obtained using a known amount of gel with the height of the peaks obtained using known dilutions of pyridoxamine phosphate. The height of the peaks caused by the aminohexyl pyridoxamine phosphate solution will be proportional to the amount of aminohexyl pyridoxamine phosphate in the solution. There is unlikely to be any free PLP in the aminohexyl pyridoxamine phosphate solution used for comparison since diaminohexane was added in excess. An approximate pyridoxamine phosphate concentration of 2 μmol/ml settled gel was estimated in this manner.

#### 6.2.1.2 Spectrophotometric analysis

The absorbance spectrum of the PMP-Sepharose 4B gel and that of the aminohexyl pyridoxamine phosphate were identical with maximum absorbance at 245 nm and 308 nm. This confirmed that the ligand had been attached to the gel. The excitation maximum (310 nm) does not quite coincide with the wavelength (308 nm) at which maximum absorbance occurred but the difference is small, 2 nm, and is probably due to machine variability.

Pyridoxamine phosphate in 0.1M NaOH has absorbance maxima at 244 nm and 312 nm. The experimental values differ slightly from these however, the difference could be due to a slight error in machine adjustment. Alternatively, the difference, particularly in the second of the two values which represents absorbance by the substituted pyridine ring, might be attributed to slight changes in charge distribution caused by the covalent bond with the diaminohexane spacer. Pyridoxamine HCl shows maximum absorbance at 245 nm and 308 nm however, the reaction conditions were such that pyridoxamine phosphate was the only pyridoxal derivative which could be formed. An approximate value for the amount of ligand bound to the gel of 2.29 μmol/ml settled gel was calculated from the extinction coefficient for pyridoxamine phosphate.

6.2.2 Chromatography on PMP-Sepharose 4B gel

The elution profile shown in figure 6.1 is representative of those obtained using the procedure described in 6.1.4. GAD activity was eluted just after the water/10 mM potassium phosphate interface. This indicated that GAD had not bound to the column, although it was retarded by the column since greater than two bed volumes of 10 mM potassium phosphate buffer were required to elute the activity completely. This interaction between the column and GAD was presumably non specific and ionic in nature. Approximately 70% of the total protein and 50% of the GAD activity was eluted in this first peak.

A second, much smaller, peak of GAD activity was eluted with 100 mM potassium phosphate. This contained 30% of the total protein and 13% of the GAD activity. Again the column probably acted as an ion exchange rather than an affinity column. This supposition is based on the fact that increased ionic strength, although only to 100 mM potassium phosphate, was sufficient for elution and also evidence presented in Section 7 that the interaction between pyridoxal phosphate and GAD, even when the pyridoxal phosphate has formed a Schiff's base with glutamate, is extremely strong in the presence of 50 mM potassium phosphate. Consequently it would be expected that the elution conditions required to overcome such a strong interaction would need to be more rigorous and probably also more specific.

Almost 100% of the protein was recovered however, 37% of the GAD activity was unaccounted for. Since GAD has proved to be particularly labile this could represent enzyme inactivation. Alternatively GAD might have bound tightly to the column and therefore not have been eluted with 100 mM potassium phosphate.

Although the ligand was attached to the gel through the aldehyde group which would probably interact with a lysine residue at the active site of GAD to form a Schiff's base, the protein should still be able to bind specifically to the ligand. It has been shown that although the aldehyde group is essential for the catalytic activity of PLP dependent enzymes with amino acid substrates, it is not essential for binding (Snell, 1970). In addition there are two reports describing chromatography of mouse brain GAD using affinity resins with a similar structure to that used here (Possani et al, 1977; Perez de la Mora et al, 1981).

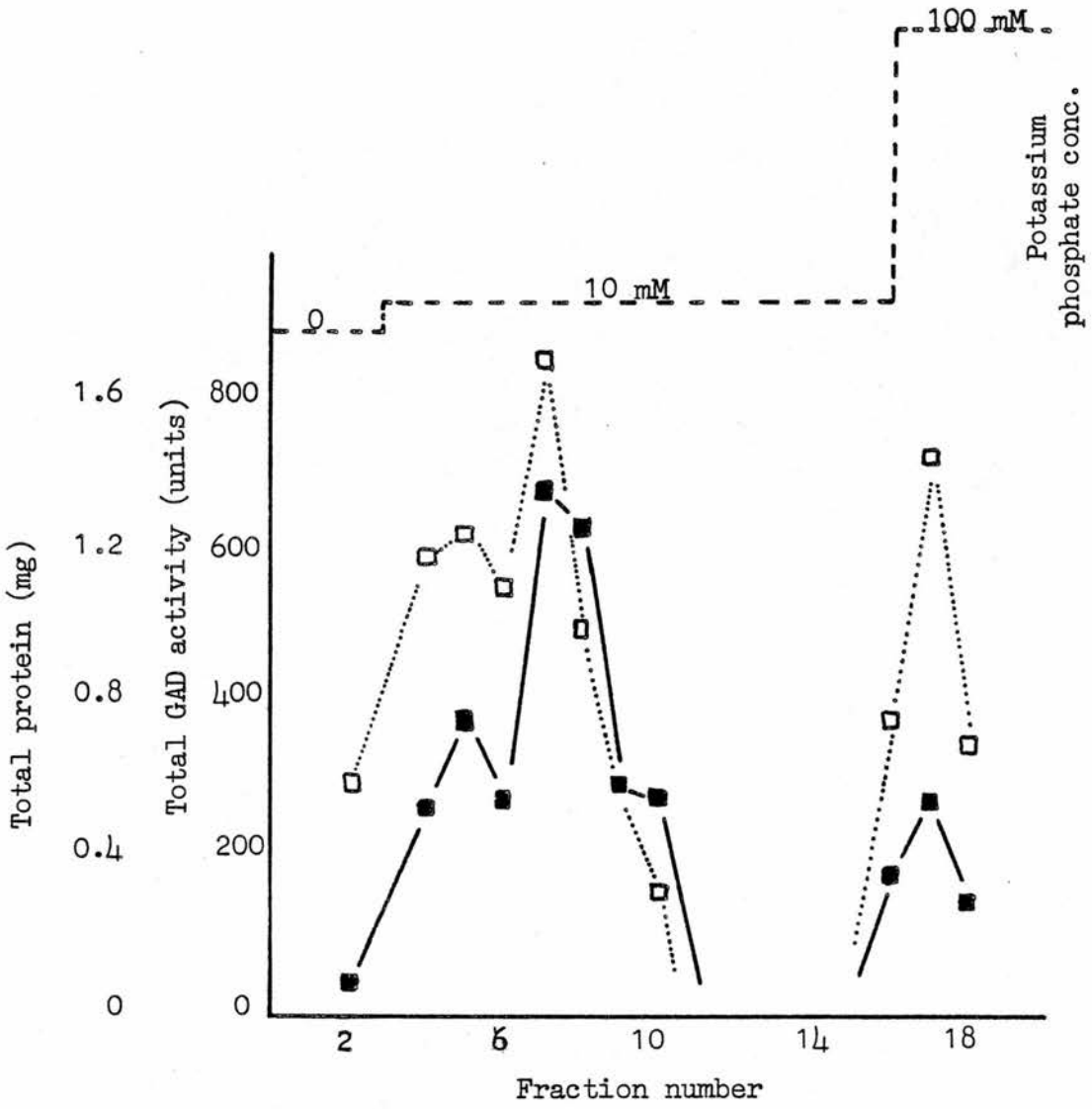



Figure 6.2 Elution profile of PMP-Sepharose 4B affinity column. Chick brain supernatant, after pretreatment with 100mM glutamate (see section 6.1.4.), was applied to a PMP-Sepharose 4B column. (- - -) indicates the potassium phosphate concentrations used for elution. Fraction size, 2ml. One unit of enzyme activity is one nmol. GABA produced /ml /hour. (▫···▫), protein concentration; (▪—▪), GAD activity.

However, there is evidence for a very tight association between GAD and PLP. It seems unlikely therefore that PLP bound to a gel matrix could displace PLP at the active site. Incubation with glutamate in imidazole acetate buffer was used to try to deplete the PLP content but, as shown in Section 7, this was not a particularly successful method. Only a small amount of any activity lost could be restored by the addition of PLP. Consequently only a small percentage could be expected to bind specifically to the column.

The GAD activity which was not accounted for might have bound to the column, although this implies that the pretreatment step had been more successful than had ever been demonstrated. Alternatively, it might represent a combination of binding and denaturation.

Despite the possibility that some GAD activity might have bound to the column it was decided not to develop affinity chromatography further. Since it had not proved to be sufficiently reproducible and the amount of GAD specifically bound to the column could only have been a very small percent of the activity present in the original supernatant. Although 37% of the activity loaded on the column was unaccounted for, this was only 12.5% of the activity in the starting material. This is probably due to enzyme inactivation caused by incubation in imidazole acetate and small losses of protein (and hence of GAD) when the fractions were collected from the Sephadex G25 columns.

This particular resin did not fulfil the criteria upon which affinity chromatography was chosen, namely, a one step method of obtaining a concentrated, much purified GAD solution from a large volume of supernatant containing a low concentration of enzyme activity. It seemed unlikely in the absence of a reliable and reversible method of removing PLP from GAD (see Section 7) that large scale preparative affinity chromatography using PLP as a ligand would be effective.

## SECTION 7: FACTORS AFFECTING THE INTERACTION OF PLP WITH GAD

### 7.1 Tissue preparation

Chicks were killed by decapitation and rats were killed by cervical dislocation. Whole chick brain or whole rat brain was homogenised 1:10 (w/v) or 1:30 (w/v) in ice cold double distilled water containing 0.1% (w/v) Triton X100 and 1 mM PMSF.

The homogenates were centrifuged at 100 000 $g_{av}$ for 1 hour and the supernatants dialysed for 24 hours, at 4°C, against 10 to 15 volumes of one of the following buffers.

(a) Phosphate buffer, which is 25 mM potassium phosphate containing 1 mM AET, pH 7.2.

(b) Imidazole acetate buffer which is 100 mM imidazole acetate containing 1 mM AET, pH 6.5.

In each case the dialysis buffer was changed twice.

### 7.2 Experimental conditions

The experimental conditions used in this section were modified from those described by Miller et al (1978).

7.2.1 Dialysed chick brain supernatant (from an homogenate, 1:10 (w/v)) in phosphate buffer was preincubated at 37°C, in the presence or absence of 10 mM glutamate, for 30 minutes. The glutamate-treated supernatant was desalted by gel filtration on a column (2 cm x 15 cm) of Sephadex G25 (equilibrated in phosphate buffer) to remove the glutamate and any PLP which may have become dissociated from the enzyme.

The supernatant which had been preincubated with glutamate and that which had been preincubated without glutamate were both assayed for GAD activity in the presence or absence of PLP. The GAD assay was performed as described in Section 4.1.2 except PLP was omitted from some of the incubations. The total incubation period for the GAD assays was 60 minutes. Samples were withdrawn at intervals (10, 20, 30, 45 and 60 minutes) so that any effect on the rate of the reaction caused by the omission of PLP could be examined.

7.2.2 The experiments described in 7.2.1 were repeated using rat brain supernatant. The total incubation period for the GAD assays was 30 minutes. Samples were withdrawn at intervals (5, 10, 20 and 30 minutes) so that any effect on the rate of the reaction caused by the omission of PLP could be examined.

7.2.3 Dialysed rat brain supernatant (from an homogenate, 1:30 (w/v)) in imidazole acetate buffer was treated exactly as described in 7.2.1, except that imidazole acetate buffer replaced phosphate buffer, until the GAD assay. The supernatant which had been preincubated with glutamate and the supernatant which had been preincubated without glutamate were both assayed in the presence or absence of PLP. Those samples which were to be assayed in the presence of PLP were preequilibrated for 5 minutes at room temperature with 1 mM PLP. The PLP concentration in the GAD assay mixture added to those samples assayed in the presence of PLP was adjusted so that the final assay concentration was 1 mM. The GAD assay incubation period was 15 minutes.

7.2.4 The experiments described in 7.2.3 were repeated using chick brain supernatant. The procedure was exactly the same except for the conditions used for preincubating the supernatant with glutamate. In these experiments the supernatant was preincubated at 37°C for 15 minutes in the presence of 10 mM, 100 mM or 250 mM glutamate or in the absence of glutamate.

In all these experiments glutamate was added as a concentrated buffered solution.

## 7.3 Results

7.3.1 These results were obtained using the experimental conditions described in 7.2.1. When a dialysed chick brain supernatant was preincubated at 37°C in the absence of added glutamate then assayed for GAD activity in the presence of 1 mM PLP the reaction was linear for 1 hour (Figure 7.1a). The rate of the reaction when the same supernatant was assayed without added PLP remained linear for 30 minutes before conditions became limiting (Figure 7.1a). The rate of the reaction over the linear region of the graph was about 15% lower when assayed in the absence of added PLP than it was when assayed in the presence of added PLP.

The linearity of the reaction (up to 30 minutes) in the absence of added PLP and the fact that the reaction rate over this period was only 15% lower than that obtained when GAD was assayed in the presence of a large excess of PLP suggests that most of the enzyme was still saturated with PLP. Since the tissue was prepared in buffer which did not contain PLP, the supernatant underwent a long period of dialysis versus PLP free buffer and the enzyme was not inactivated by gel filtration using Sephadex G25, it can be concluded that PLP must be very tightly bound to the enzyme. This agrees with the findings of Miller *et al* (1977) who reported that the bond between PLP and rat brain GAD was stable to both dialysis and gel filtration.

When a dialysed chick brain supernatant was preincubated with 10 mM glutamate followed by gel filtration on Sephadex G25 (see 7.2.1) and then assayed for GAD activity with 1 mM PLP or without added PLP the results obtained (Figure 7.1b) were similar to those described for a supernatant which had not been preincubated with glutamate. However, the omission of PLP had a much greater effect on the rate of the GAD reaction using glutamate-treated supernatant than it did using non glutamate-treated supernatant. Over the linear region of the plot there was a 29% reduction in the rate of the reaction using a glutamate-treated supernatant (Figure 7.1b) compared with a 15% reduction using an non glutamate-treated supernatant (Figure 7.1a) when PLP was omitted.

There was a lower level of GAD activity in the glutamate-treated supernatant than in the non glutamate-treated supernatant, under the same conditions, implying that the glutamate-treated enzyme might be less saturated with PLP and therefore, that glutamate had had an effect on the PLP bound to the enzyme.

Figure 7.1. Comparison between the GAD activity in untreated, dialysed chick brain supernatant with that in dialysed chick brain supernatant after preincubation with 10 mM glutamate.

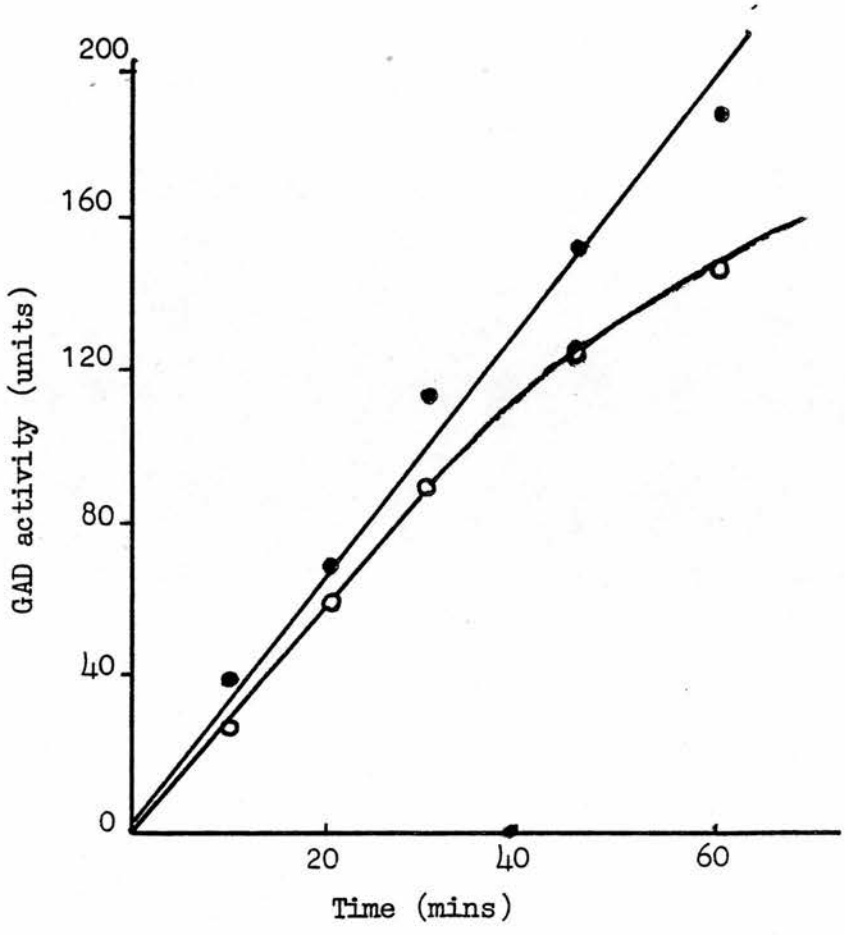
Samples assayed with (●-●) and without (O-O) the addition of 1 mM PLP. Dialysis was for 24 hours at 4°C against 25 mM potassium phosphate containing 1 mM AET, pH 7.2.

(a) GAD activity in dialysed chick brain supernatant which had been prewarmed at 37°C for 30 minutes before assay.

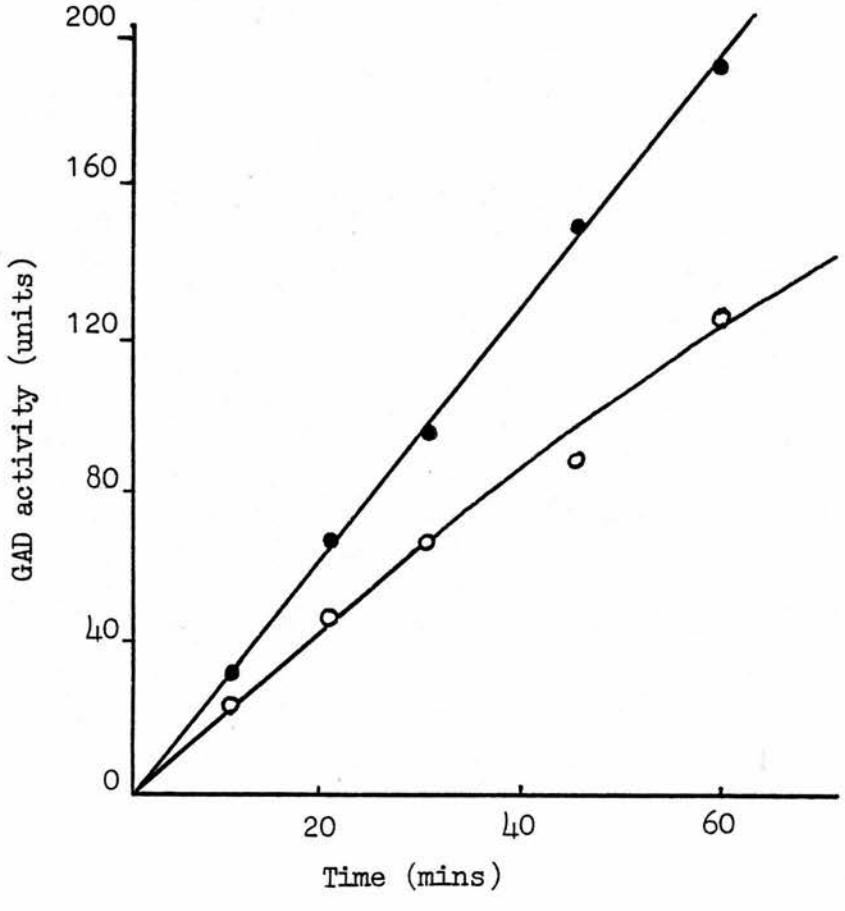
(b) GAD activity in dialysed chick brain supernatant which had been preincubated with 10 mM glutamate at 37°C for 30 minutes before assay.

Experimental details are contained in Section 7.2.1. 1 unit enzyme activity = 1 nmol GABA produced/mg protein. Data are the means from 2 sets of experiments with triplicate determinations.

(a)


200
160
120
80
40
0
GAD activity (units)
20
40
60
Time (mins)

(b)


200
160
120
80
40
0
GAD activity (units)
20
40
60
Time (mins)

However, the effect of 10 mM glutamate on the PLP bound to chick brain GAD was small under these conditions since the enzyme still appeared to be greater than 70% saturated with PLP. The dialysed but non glutamate-treated supernatant appeared to be 85% saturated. Per cent saturation is used to express the activity which remains after the various treatments and was defined by Miller et al(1978) as the activity obtained in the absence of added PLP expressed as a percentage of the activity obtained with a (presumed) saturating concentration of PLP.

7.3.2 These results were obtained using the experimental conditions described in 7.2.2. The previous experiment was repeated using rat brain supernatant and produced very similar results (Figure 7.2) to those obtained with chick brain supernatant (Figure 7.1). The rate of reaction over the first 30 minutes, when PLP was omitted, was 16% lower using a non glutamate-treated supernatant and 30% lower using a glutamate-treated supernatant. Preincubation with 10 mM glutamate in phosphate buffer at 37°C caused a small reduction in GAD activity when the enzyme was assayed without PLP. The effect on chick and rat brain GAD appeared similar.

7.3.3 Miller et al (1978) described substrate-promoted dissociation of PLP from rat brain GAD in 100 mM imidazole acetate, pH 6.5, rather than in phosphate buffer. Therefore, the previous experiments were repeated using rat brain supernatant and the experimental conditions described in 7.2.3. Preincubation with 10 mM glutamate resulted in a considerable reduction in GAD activity when the supernatant was assayed in the absence of added PLP (Figure 7.3). The GAD activity in the glutamate-treated supernatant assayed without added PLP was 54% lower than that in the non glutamate-treated supernatant assayed under the same conditions and 79% lower than that in the non glutamate-treated supernatant assayed with PLP. The addition of PLP to the glutamate-treated supernatant caused a 19% increase in GAD activity to 40% of that in the non glutamate-treated supernatant assayed with PLP. If the GAD in the untreated supernatant assayed in the presence of PLP is regarded as fully saturated then preincubation with 10 mM glutamate caused an apparent 79% decrease in saturation. However, a 19% increase in enzyme activity to only 40% of the original GAD activity was obtained by the addition of PLP rather than the expected

Figure 7.2. Comparison between the GAD activity in untreated, dialysed rat brain supernatant with that in dialysed rat brain supernatant after preincubation with 10 mM glutamate

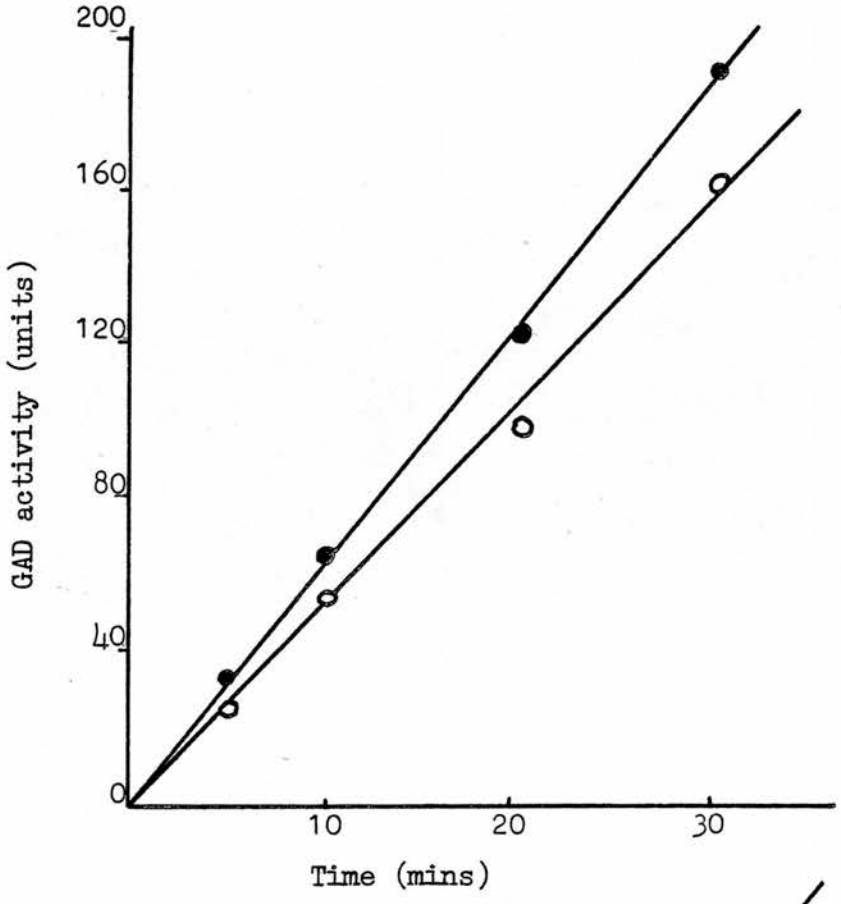
Samples assayed with (●–●) and without (O–O) the addition of 1 mM PLP. Dialysis was for 24 hours at 4°C against 25 mM potassium phosphate containing 1 mM AET, pH 7.2.

(a) GAD activity in rat brain supernatant which had been prewarmed at 37°C for 30 minutes before assay.

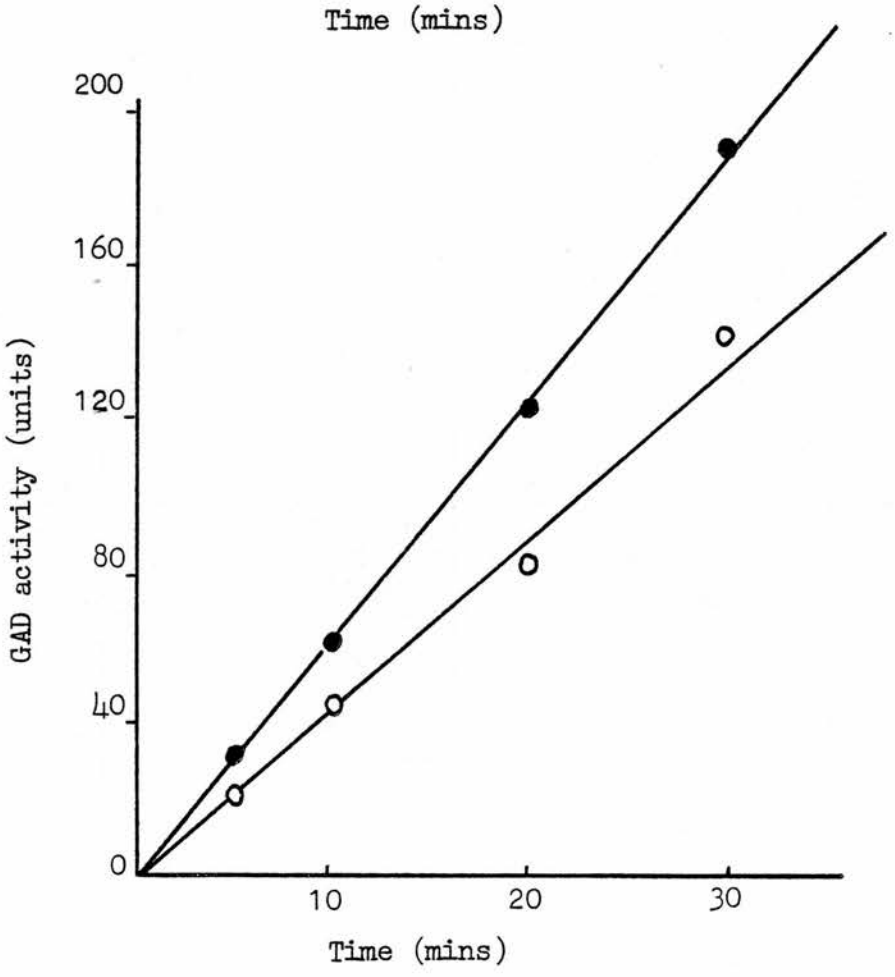
(b) GAD activity in rat brain supernatant which had been preincubated with 10 mM glutamate at 37°C for 30 minutes before assay.

Experimental details are contained in Section 7.2.2. 1 unit enzyme activity = 1 nmol GABA produced/mg protein. Data are the means from 2 sets of experiments, triplicate determinations.

(a)


200
160
120
80
40
0
GAD activity (units)
10
20
30
Time (mins)

(b)


200
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GAD activity (units)
10
20
30
Time (mins)

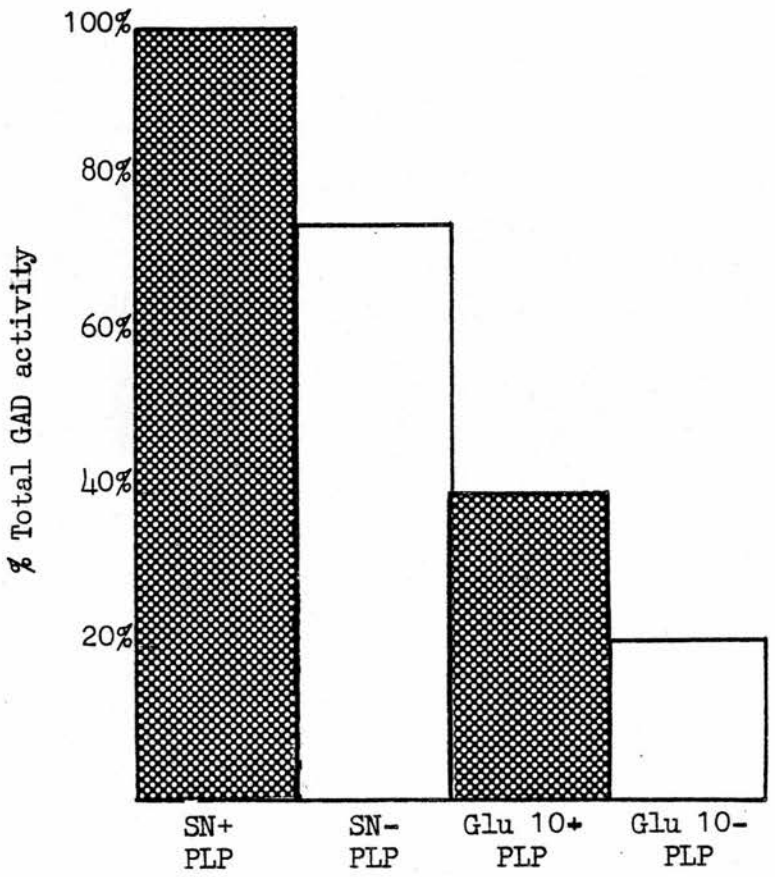



Figure 7.3 Comparison between the GAD activity in untreated, dialysed rat brain supernatant (SN) with that in dialysed rat brain supernatant after preincubation with 10 mM glutamate (Glu 10). Samples assayed with (SN+PLP, Glu 10+PLP) and without (SN-PLP, Glu 10-PLP) 1mM PLP. Dialysis was for 24 hours at 4°C against 100 mM imidazole acetate containing 1mM AET, pH 6.5. Experimental details are contained in section 7.2.3. The data are the means from 2 sets of experiments expressed as percentages of the GAD activity (nmol. GABA produced / mg protein) in untreated rat brain supernatant assayed with 1mM PLP (SN+PLP). Each determination was made in triplicate.

complete recovery. This indicated that the procedure caused a large amount of irreversible inactivation of the enzyme. In addition it is interesting to note that the GAD activity in the non glutamate-treated supernatant assayed without PLP is 75% of the control value (Figure 7.3) compared with 85% using phosphate buffer (Figure 7.2). If these effects on activity are due to substrate-promoted dissociation of the cofactor, it would appear that a large percentage of the dissociation was irreversible since, in these experiments, addition of PLP did not regenerate much GAD activity.

7.3.4 Dialysed chick brain supernatant, in imidazole acetate buffer, was preincubated with 10 mM, 100 mM and 250 mM glutamate before it was assayed for GAD activity with and without added PLP (see Figure 7.4). Experimental details are described in Section 7.2.4. Data was compared using the Students t test.

Preincubation with 10 mM, 100 mM and 250 mM glutamate caused a decrease in GAD activity in supernatant assayed without added PLP of 28%, 42% and 26% respectively when compared with the activity in a non glutamate-treated supernatant assayed with PLP (see Figure 7.4). This difference was statistically significant. However, under these experimental conditions the GAD activity in a non glutamate-treated supernatant assayed without added PLP was reduced by 30%, again statistically different from the control value. When this value was compared with those for GAD activity in glutamate treated supernatants assayed without PLP the difference was not statistically significant.

Addition of PLP to the glutamate-treated supernatants failed to restore GAD activity to the value of the non glutamate-treated supernatant assayed with added PLP. In the cases of the supernatants treated with 10 mM and 100 mM glutamate the GAD activity assayed with added PLP was not significantly different from that assayed without PLP. Addition of PLP to the supernatant treated with 250 mM glutamate did cause a significant increase in activity however, this was still significantly lower than the GAD activity in the non glutamate-treated supernatant assayed with PLP.

From these results it would appear that preincubation with glutamate in imidazole acetate buffer may have caused a small amount of mostly irreversible inactivation of GAD. Since there was only 70% of the control GAD activity present in the non glutamate-treated supernatant

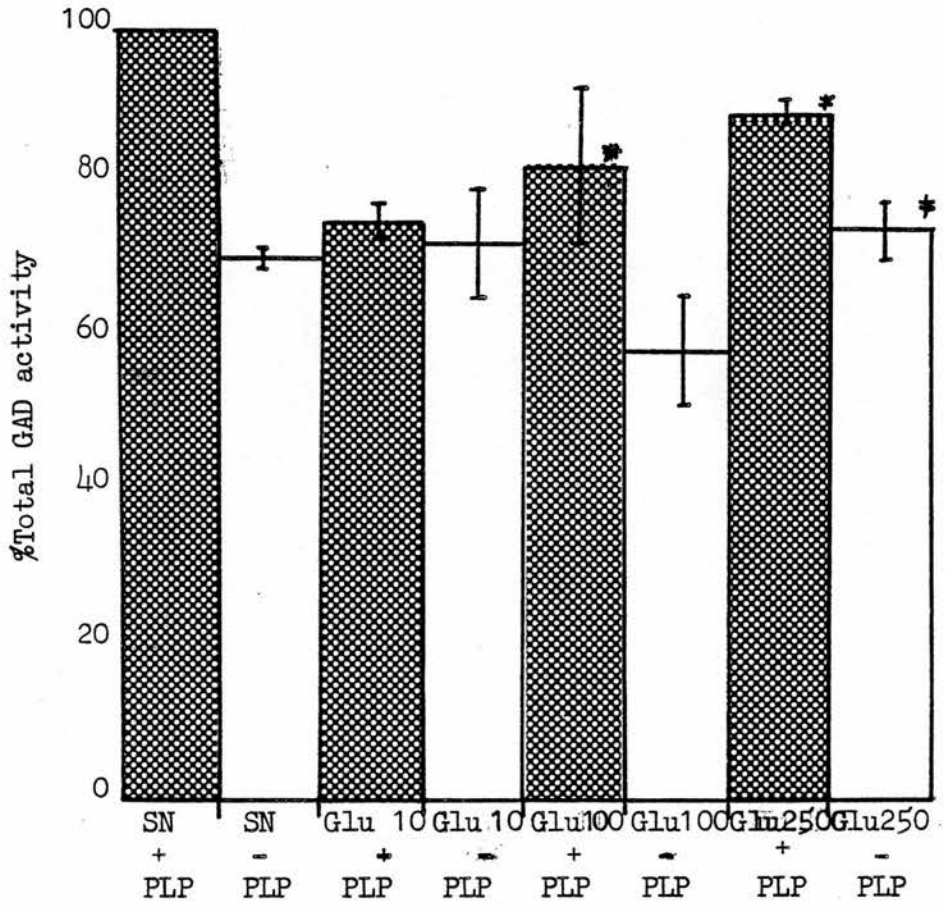



Figure 7.4 Comparison between the GAD activity in untreated, dialysed chick brain supernatant (SN) with that in dialysed chick brain supernatant after preincubation with 10 mM (Glu 10), 100 mM (Glu 100) or 250 mM (Glu 250) glutamate. Samples assayed with (+PLP) and without (-PLP) 1 mM PLP after preincubating or prewarming for 15 minutes at 37°C. Dialysis was for 24 hours at 4°C against 100 mM imidazole acetate buffer containing 1 mM AET, pH 6.5. Experimental details are in section 7.2.4. Results are expressed as percentages of the GAD activity ( nmol. GABA produced /mg protein ) in untreated chick brain supernatant assayed with PLP. Each value is the mean of 3 experiments $\pm$ s.e.m. Each experiment used 2 birds with triplicate determinations. The data were compared using the Student's t test. * indicates that the value was not significantly different from (SN+PLP) $p > 0.005$, d.f. = 4. # indicates that the values are significantly different from each other $p < 0.005$ d.f. = 4.

assayed without PLP compared with 85% when the experiment was performed using phosphate buffer it can be inferred that either phosphate buffer is protective or that imidazole acetate buffer causes some denaturation.

Preincubation with 10 mM glutamate followed by assay for GAD activity without added PLP resulted in the same level of GAD activity as in the untreated supernatant, assayed under the same conditions, implying that much of the effect was promoted by the buffer conditions. The effect was not readily reversible, addition of PLP increased GAD activity by 3% (Figure 7.4). This result is interesting since preincubation with 10 mM glutamate in imidazole acetate buffer resulted in a marked decrease in activity for rat brain GAD (Figure 7.3).

Preincubation with 250 mM glutamate caused a decrease in GAD activity of 26% compared with the control value (Figure 7.4). The addition of PLP resulted in the recovery of 15% of the enzyme activity. The most marked effects were obtained by preincubating the supernatant with 100 mM glutamate. GAD activity was reduced by 42% by preincubation with 100 mM glutamate followed by assay without added PLP. Addition of PLP caused a recovery of activity of 24%.

Of the glutamate concentrations used to try to promote dissociation of PLP from chick brain GAD, only 100 mM glutamate in imidazole acetate reduced the enzyme activity, in the absence of PLP, to less than the non glutamate treated control under the same conditions. In addition the effect was to some extent reversible. There did not appear to be substrate-promoted dissociation of PLP from either chick or rat brain GAD using phosphate buffer.

## 7.4 Discussion

The effects on GAD activity observed when chick and rat brain supernatants were incubated with glutamate in imidazole acetate buffer are somewhat difficult to interpret. The fact that pretreatment with 10 mM glutamate at 37°C in imidazole acetate buffer had a much greater effect on rat brain GAD (Figure 7.3) than pretreatment with the same, or higher concentrations of glutamate, had on chick brain GAD (Figure 7.4) under the same conditions may be consistent with the idea that chick and rat brain GAD are structurally different (Saito *et al* 1974). The results obtained strongly suggest that the buffer played an important role in causing the effects observed after pretreatment with glutamate.

Imidazole has a ring structure comprised of carbon atoms and two nitrogen atoms and at pH 6.5 would exist both in the protonated form and as the non ionised free base. The charge distribution of the protonated imidazole ion may be similar enough to that of the pyridoxal ring to dislodge the cofactor under certain conditions, for example, when it is no longer bound via a Schiff's base to the protein but has formed a Schiff's base with glutamate. Differences in the amino acid sequence in the vicinity of the active site between rat and chick brain GAD may result in PLP being held more strongly in the latter case and therefore being more difficult to dislodge.

There is evidence that imidazole, in the protonated state, can affect the conformation of a protein. Shaltiel *et al* (1966 & 1969) used imidazole citrate at pH 6 and pH 7 as a "deforming" buffer in the removal of PLP from phosphorylase *b*. The data suggested that the imidazole ion, rather than the free base, caused localised denaturation of the protein exposing the PLP which could then be removed by a carboxyl reagent although at higher concentrations of imidazole a carbonyl reagent was unnecessary. The denaturation was reversed by the removal of the imidazole.

A similar, although not completely analogous, situation might arise with GAD. Since PLP is a cofactor participating in the decarboxylation of glutamate it must be accessible to glutamate and therefore it is unlikely to be inaccessible to other small molecules such as hydrazides which will also react with the aldehyde group in contrast with phosphorylase *b*. Consequently, although the imidazole

ion may not interact with the active site itself, the environment of the active site may be influenced by interactions with other regions of the molecule. Since it is probable that the amino acid sequence of chick and rat brain GAD differ (Saito et al 1974) the imidazole acetate buffer might distort one enzyme more than the other. Rat brain GAD might therefore be more susceptible than chick brain GAD.

It would be necessary to assay for the presence of PLP to be certain that the alterations in GAD activity were due only to the removal of PLP. If the GAD activity was reduced due to reversible substrate-promoted dissociation as has been suggested (Miller et al 1978) it should have been possible to regenerate almost 100% activity by the addition of PLP. However, that did not prove to be the case in these experiments. It has been shown (Shaltiel et al 1966) that if too high a concentration of imidazole is used (for phosphorylase b, at this pH, between 0.6M and 0.8M) the protein may become denatured rather than "deformed". It is possible that in these experiments the concentration of imidazole was approaching that critical concentration for GAD and therefore that a significant amount of PLP was lost irreversibly.

The experiments described in this section were designed to exploit the phenomenon of substrate-promoted dissociation, reported by Miller et al (1978), to deplete chick brain GAD of PLP, prior to affinity chromatography. There has been controversy as to the strength of the association between GAD and PLP. In vivo evidence is contradictory and has suggested that PLP may be loosely bound to the active site (Baxter 1969) so that GAD might be regulated by cofactor availability, but also that PLP may be tightly bound to GAD since enzyme activity is unaffected by changes in PLP concentration in vivo (Gey & Georgi 1974). It has been postulated, from in vitro studies, that neuronal GAD may have two types of binding site, one which binds the cofactor tightly, the other weakly (Tapia & Sandoval 1971; Bayon et al 1977(a) & (b)) whereas Miller et al (1978) described very tight association between GAD and PLP in the absence of glutamate, with dissociation from the active site in the presence of glutamate.

The evidence presented in this section is in agreement with the proposal that the enzyme and cofactor are tightly associated. However, the data regarding the possibility of substrate-promoted dissociation

conflict with the previous report (Miller et al 1978). There is no good evidence for substrate-promoted dissociation of PLP from GAD in phosphate buffer (Figures 7.1 & 7.2). In imidazole acetate buffer the evidence is against rather than for substrate-promoted dissociation of PLP from chick brain GAD (Figure 7.4). In the case of rat brain GAD, although enzyme activity was reduced by the treatment described (Figure 7.3) to similar levels as previously reported (Miller et al 1978), in contrast only a small percentage could be regenerated by the addition of PLP. Although many of these observations have been attributed to buffer effects, this does not preclude any facilitation of the removal of PLP by the formation of a Schiff's base with glutamate but suggested that under these unphysiological conditions this may be a secondary effect.

A $K_m$ for PLP for chick brain GAD

Data obtained from these experiments highlight the difficulties involved in the estimation of a $K_m$ for PLP, described in Section 5. It did not prove possible to determine an apparent $K_m$ for PLP for GAD using chick brain cytosol since the enzyme had apparently remained saturated with its cofactor. This is corroborated by the evidence for a very strong association between PLP and chick brain GAD in phosphate buffer presented in this section (See 7.3.1).

Values for a $K_m$ for PLP have been reported for mouse, rat and human brain GAD of 0.05 μM (Wu et al 1973), 0.5 μM (Maitre et al 1978) and 0.1 μM (Blindermann et al 1978(a)) respectively using the purified protein. Blindermann et al (1978(a)) described a method of preparing the apoenzyme which was similar in principle to that described in this section but substituted GABA, which acted as a weak competitive inhibitor, for the substrate glutamate. Although there was no "deforming" agent present it is possible that the rigours of the purification schedule may have slightly altered the conformation of the protein so that the cofactor was not held so tightly in place. In addition the ionic interactions between PLP and the protein would probably not be as strong at the pH used (pH 5) to prepare the apoenzyme consequently the addition of a high concentration of a competitive inhibitor might be sufficient to promote binding and allow the removal of the cofactor. Wu et al (1973) make no mention of the necessity of preparing the apoenzyme however, the purification protocol used contained several ammonium sulphate precipitations. Evidence from other enzymes (Snell 1970) showed that ammonium sulphate precipitation resulted in the loss of PLP.

Although attempts were made to establish an apparent $K_m$ for PLP for chick brain GAD, a stable, pure enzyme preparation is necessary for an accurate value to be determined. Using a crude system such as chick brain cytosol it is not possible to control all the factors which might affect the $K_m$. Assuming that the system could be completely depleted of PLP there would be competition for the cofactor by other PLP requiring enzymes for a total PLP concentration which might be as low as 0.01 uM. Another factor which would significantly affect the level of available PLP at such a low concentration is that PLP will readily and non-specifically bind to protein (Snell 1970), albeit with low affinity.

Finally, steady state kinetic theory assumes that the rate of association and dissociation of cofactor or substrate is not slow in comparison with the overall rate. However, evidence suggests that dissociation of PLP is at least slow and there is no definitive evidence that, in vivo, dissociation does occur. Consequently values for a $K_m$ for PLP may not be meaningful and may depend on other, as yet unidentified, factors.

Regulation of enzyme activity

Enzyme activity can be regulated not only by substrate or cofactor availability or by binding with effector molecules which alter enzyme activity but also by a variety of cellular mechanisms which affect transcription or translation of proteins. The manner in which GABA synthesis is controlled and GAD activity regulated has not been elucidated. In view of the high glutamate concentration throughout the brain (approximately 3 µmol/g wet weight, Levi & Morisi 1970) it is doubtful that the levels would fluctuate sufficiently for substrate availability to control GABA synthesis. Preliminary experiments have been carried out by several workers in an attempt to uncover a control mechanism for GAD.

Miller et al (1978) suggested that association and dissociation of PLP might play a role in regulating GABA synthesis. They postulated that the high concentration of glutamate in brain prevented saturation of the enzyme by causing substrate promoted dissociation of the PLP and necessitating a constant supply of PLP to maintain activity. This was modified to include the effects of adenine nucleotides on GAD activity by Seligman et al (1978) who followed up earlier reports (Tursky et al 1970)

that ATP inhibited GAD non-competitively with respect to both substrate and cofactor in a manner which was dependent on PLP concentration. On the basis of data showing that ATP, ADP and the magnesium complexes of ATP and ADP were potent inhibitors of GAD in dialysed preparations of rat brain cytosol it was proposed that, in the absence of ATP, available PLP reactivated enzyme which had been inactivated by substrate promoted dissociation. This reactivation could however, be blocked by ATP (at physiological concentrations) which bound to the protein causing a conformational change so that the active site was blocked. In the presence of high concentrations of PLP, 100 μM, which is several fold higher than physiological concentrations (physiological PLP concentrations, approximately 15 μM, Bilodeau 1965) this ATP inhibition was lifted since reactivation occurred as rapidly as the apoenzyme was formed (Seligmann et al 1978), see Figure 7.5. The observation that, in vivo, GAD is only partially saturated with PLP despite a concentration which appeared high enough for full activation (15 μM) and that a post-mortem activation of GAD was accompanied by a decrease in ATP concentration (Miller et al 1977) was presented as corrobative evidence.

However, more recently Martin et al (1980) carried out similar experiments to those previously described (Miller et al 1978) replacing rat brain cytosol with partially purified hog brain GAD. The results obtained were similar to those previously reported however, inhibition of GAD by ATP was apparently PLP-independent, in contrast with previous observations (Seligmann et al 1979).

Kinetic experiments using mouse brain cytosol produced data which were interpreted as being consistent with two types of PLP binding site, one which bound PLP tightly and was therefore unaffected by exogenous PLP levels and the other which was only weakly associated with PLP and therefore dependent on the exogenous PLP concentration (Tapia & Sandoval 1971; Bayon et al 1977(a) & (b)). This would result in a constant basal level of GABA synthesis with increased synthesis in the presence of increased levels of PLP. On the basis of this the kinetic model shown in Figure 7.6 was suggested.

The model for the control of GAD activity proposed by Miller et al (1978) and modified by Seligmann et al (1978) relies on the ready dissociation of PLP in the presence of high, but still physiological

Figure 7.5. A mechanism proposed for the α-decarboxylation of glutamate by GAD (from Seligmann et al 1978)

PLP = pyridoxal phosphate, G = glutamate, E = apoenzyme. Normally decarboxylation occurs to produce GABA however, a small fraction of the time the E-PLP-G complex may dissociate resulting in progressive inactivation in the absence of PLP. In the presence of PLP the reaction rate remains constant. ATP competes with PLP for the enzyme although this competition is overcome by high PLP concentrations. See text, Section 7 for discussion.

Figure 7.6. A kinetic model proposed for the α-decarboxylation of glutamate by GAD (from Bayon et al 1977(b))

It is postulated that GAD possesses a free PLP dependent and free PLP independent catalytic activity. In the latter case PLP is very tightly bound to the catalytic site. See text, Section 7. for discussion. SA = glutamate-PLP Schiff's base, A = free PLP, S = free glutamate, P = reaction products, E = apoenzyme, Ea = enzyme with free PLP independent catalytic activity

Figure 7.7. A hypothetical kinetic model for the α-decarboxylation of glutamate by GAD

Normally decarboxylation occurs to produce GABA however, a small fraction of the time a "mistake" may occur resulting in the inactive E-PMP complex. Exogenous PLP, possibly by transamination could cause regeneration of PLP at the active site. See text, Section 7. for discussion. PLP = pyridoxal phosphate; PMP = pyridoxamine phosphate; G = glutamate.

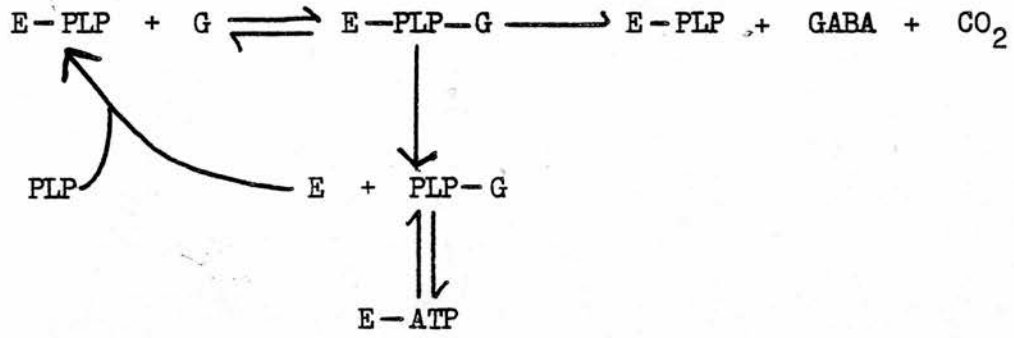



Figure 7.5

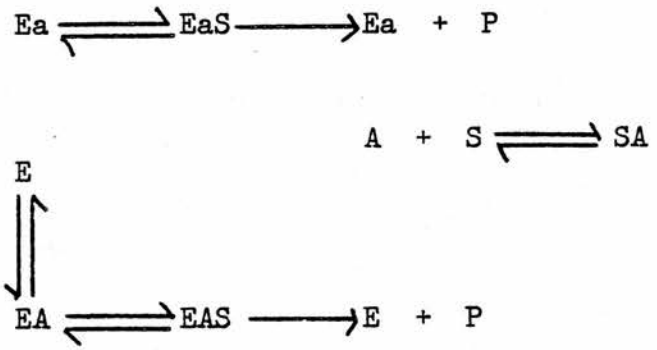



Figure 7.6

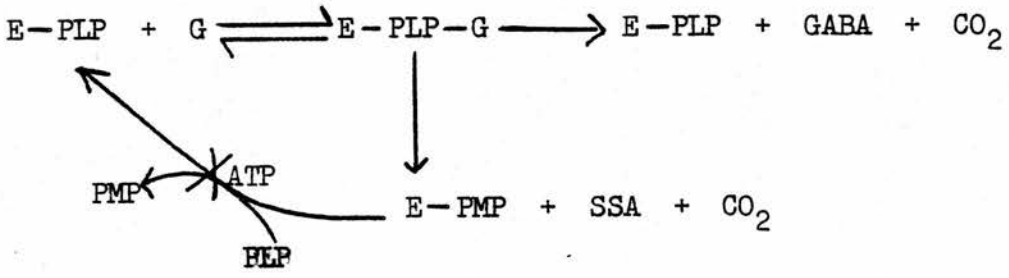


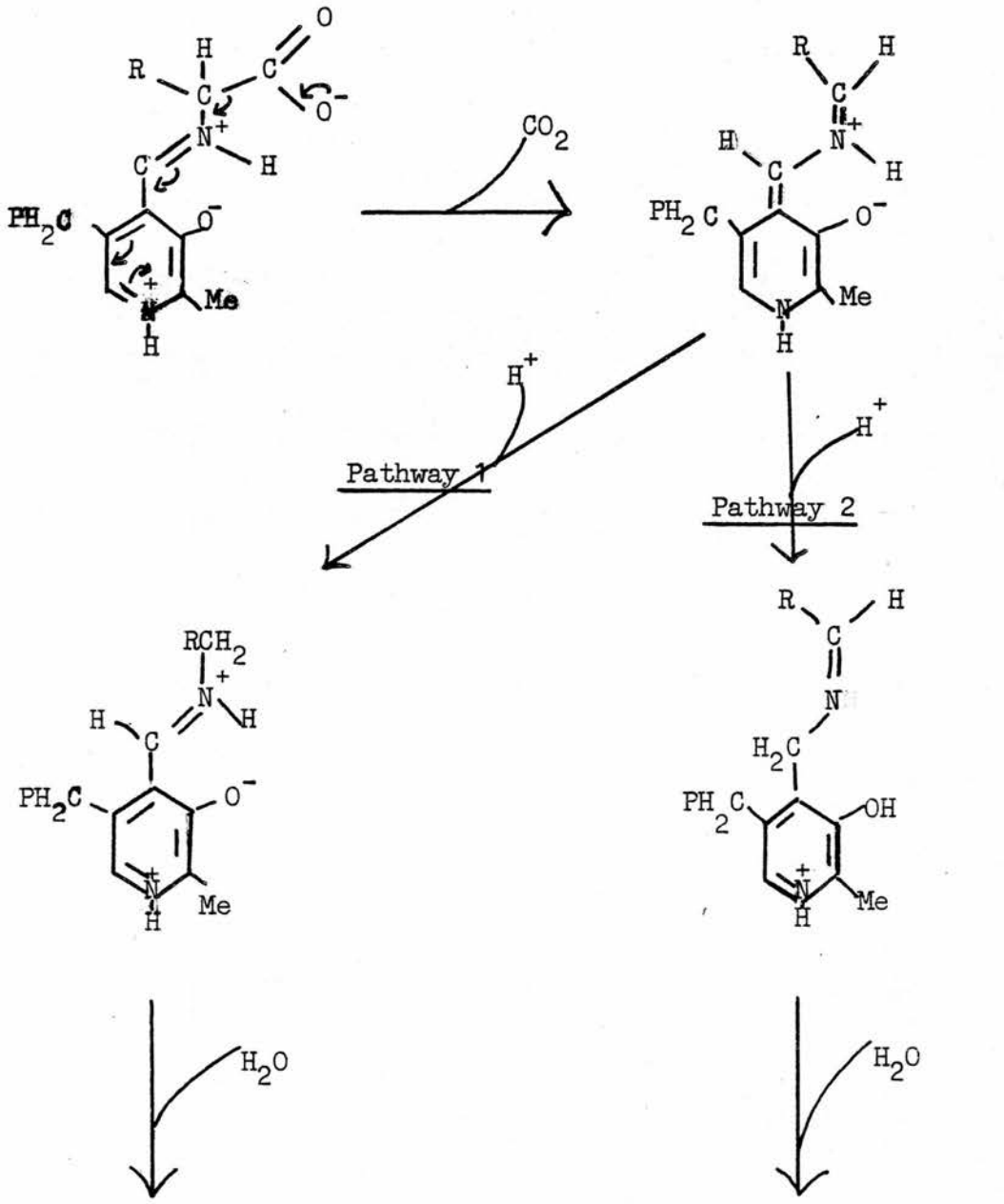

Figure 7.7

concentrations of glutamate. However, evidence presented in this section suggests that PLP may not readily dissociate from GAD under physiological conditions. An alternative mechanism which would fit the observations made here and be compatible with those made by other groups (Miller et al 1978; Seligmann et al 1978; Martin et al 1980) is outlined in Figure 7.7. Briefly, the PLP would remain tightly bound to the enzyme throughout the reaction by virtue of strong interactions involving the phosphate group and the methyl group (Snell 1970) irrespective of the glutamate concentration. This raises the question of why, if PLP never dissociates from the enzyme, exogenous PLP is necessary to maintain activity.

Figures 7.1 and 7.2 show that even in the absence of PLP the inactivation of the enzyme is relatively slow. If the inactivation were due to the loss of cofactor at each turnover of the enzyme, it would be expected that enzyme inactivation would be much more rapid. The data is more indicative of the cofactor slowly but progressively becoming unavailable.

One possible explanation is as follows. Figure 7.8 shows a schematic diagram of α-decarboxylation of an amino acid. GAD catalyses the α-decarboxylation of glutamate to produce GABA, in this diagram pathway 1. However, as the diagram indicates, after decarboxylation the proton could be added to the pyridoxal carbon atom which would result, after hydrolysis, in the production of succinic semialdehyde and pyridoxamine, pathway 2. Presumably pathway 2 must be in some way unfavourable otherwise GABA and succinic semialdehyde would be produced in equivalent amounts. However, if for some reason occasionally a "mistake" was made this would result in the formation of pyridoxamine phosphate which would be tightly bound to the enzyme by virtue of its phosphate and methyl groups and would result in slow, progressive inactivation in the absence of added cofactor since the pyridoxamine phosphate at the active site would preclude further decarboxylation.

Transamination with exogenous PLP might be a means of regenerating PLP at the active site without dissociation of the cofactor. Such a transamination might be catalysed enzymatically however, non-enzymatic transamination will occur readily at neutral pH (Snell 1958) which might also result in the regeneration of PLP at the active site.




$RCH_2NH_2$ + PLP

GABA — pyridoxal phosphate

RCHO + PMP

succinic semialdehyde — pyridoxamine phosphate

Figure 7.8 Schematic diagram showing α decarboxylation of an amino acid. See section 7.4. for discussion.

Inhibition of GAD by ATP can readily be incorporated into this model. It could prevent regeneration of PLP either by inhibiting an enzyme catalysed transamination or by binding to GAD and either blocking the active site or causing a conformational change so that the active site becomes inaccessible.

Although this suggested mechanism is speculative it does fit the observations, made during these and other experiments (Miller et al 1978; Seligmann et al 1978) and can be modified to include the influence of other factors such as ATP. It does not take into account however, the observations of Bayon et al (1977(b)) who proposed two binding sites with differing affinities for PLP.

Although a number of possible mechanisms for the control of GABA synthesis have been described, these would appear to be responsible for control of a basal level of GABA synthesis in response to the general metabolic state of the cell. It might be thought, since there is no evidence for specific intracellular storage sites for GABA, that during periods of increased neuronal activity there might be a specific mechanism responsible for causing a rapid increase in the rate of GABA synthesis. Preliminary experiments using mouse brain cytosol suggested that phosphorylating conditions increased GAD activity (Sze 1978). These results apparently contradict other observations (Tursky 1970; Seligmann et al 1978; Martin et al 1980) however pretreatment with 5 mM $Ca^{2+}$ before incubation under phosphorylating conditions suggests that the activation was $Ca^{2+}$-dependent.

Analogy was drawn with tryptophan hydroxylase (tryptophan-5-monooxygenase EC.1.14.16.4) for which there was evidence of activation through the action of a cyclic nucleotide-dependent protein kinase. Subsequently activation of tyrosine hydroxylase by a $Ca^{2+}$-dependent, calmodulin-dependent protein kinase in the presence of $Mg^{2+}$/ATP has been demonstrated (Yamauchi et al 1981). There is no further evidence for such a mechanism for activation of GAD although Miller et al (1980) have reported a $Ca^{2+}$-dependent increase in GAD activity associated with synaptosomal depolarisation.

A $Ca^{2+}$-dependent mechanism of activation of GAD, possibly through phosphorylation, presents an interesting model for the regulation of GAD activity enabling a rapid response of increased GABA synthesis on

stimulation of the neurons. Such a mechanism would link the regulation of GAD activity, neuronal activity and $Ca^{2+}$-dependent release of GABA (De Belleroche & Bradford 1972; Levy et al 1973; Redburn et al 1976).

It has also been proposed that coupled synthesis-secretion of GABA occurs (Tapia et al 1975). This has been linked with the observation that GAD binds to synaptic membranes and liposomes in the presence of $Ca^{2+}$ (Fonnum 1968; Covarrubias 1978 & 1980). Consequently $Ca^{2+}$-dependent activation of GAD may be involved with coupled synthesis-secretion rather than depolarisation-secretion of GABA, or both.

It should be possible to reconcile conflicting data to a limited extent. It is possible that interactions between GAD, glutamate, PLP and ATP provide a means of controlling the basal level of GABA synthesis which could maintain ionic inhibition by leakage into the synaptic cleft (or by coupled synthesis-secretion coupling) and also be involved in general metabolism through the GABA shunt (see 1.6). In addition some other factor, for example $Ca^{2+}$-dependent phosphorylation, might be responsible for changes in GAD activity in response to physiological changes.

The mechanism by which GABA is released into the synaptic cleft is not known. It is possible that tonic inhibition is maintained by non-specific leakage of GABA and that coupled synthesis-secretion represents the mechanism of release in response to depolarisation with the influx of $Ca^{2+}$ causing increased association of GAD with the synaptic membrane resulting in increased synthesis and release.

Much of this discussion has been speculative based on a limited amount of evidence. However, strongly illustrated is the lack of any strong, unequivocal evidence in favour of a particular control mechanism for GAD. The in vivo evidence is contradictory (undoubtedly due to the complexity of the tissue and difficulties associated with the compartmentalisation of GABA metabolism) and most of the in vitro evidence has been obtained using crude, often unphysiological conditions, where any interactions or factors affecting enzyme activity cannot be adequately predicted, monitored or controlled. A consequence of this is ambiguous or contradictory results which may simply be the result of differing tissue preparation or buffer conditions. In addition the use of unphysiological buffers and concentrations can make it difficult to relate in vitro observations to the in vivo situation.

## SECTION 8: IMMUNOLOGY

### 8.1 Methods

#### 8.1.1 Double immunodiffusion (Ouchterlony Technique)

Noble agar, 2% (w/v) in water, was mixed 1:1 (v/v) with 0.1M sodium barbitone buffer which was adjusted to pH 8.4 with 1M HCl and contained 0.1% (w/v) sodium azide as a bacteriostat (Dawson et al 1979). This was stored at 4°C until use. The agar was melted in a boiling water bath and applied to clean glass slides (or plates), which were on a levelling table to ensure an even depth of agar throughout. The agar was applied using a warm pipette, 2 ml per slide (2.6 cm x 7.6 cm) and 14 ml per glass plate (8.2 cm x 8.2 cm). Seepage of molten agar under the slide or plate was prevented by placing them on small washers (1 mm deep). The pattern was cut in the agar using a template. The samples and appropriate antisera were applied to the wells, 2.5 µl per well when using the small glass slides, 5 µl per well when using the glass plates, and the slides or plates placed in a sealed moist chamber and left for 24 hours at room temperature for the precipitin arcs to develop.

#### 8.1.2 Washing and staining immunodiffusion plates

Unprecipitated protein was removed by washing the plates in 0.9% (w/v) sodium chloride (containing 0.1% sodium azide) for 48 hours at room temperature. The sodium chloride was changed after 24 hours. Salt was removed from the agar by washing in distilled water (containing 0.1% sodium azide) for 48 hours, at room temperature, with 1 change after 24 hours. The agar was dried down by laying a piece of wet Whatman number 1 chromatography paper over the agar and leaving at room temperature overnight or placing in an oven at 37°C. When the process was complete the paper was dampened with distilled water and peeled off. Any paper still adhering to the agar was removed using a wet, soft bristled brush.

The plate was soaked in 2% (v/v) acetic acid for 10 minutes and the precipitated protein stained by immersing the plate for 10 minutes in 0.5% (w/v) amido black in methanol-glacial acetic acid (9:1 v/v). To destain the plate was washed in methanol-glacial acetic acid (9:1 v/v) until the background was clear then dried in an oven at 37°C.

### 8.1.3 Preparation of solid phase antibody

Oxidation of the gel

Sephacryl S300 (Superfine), 15 ml settled volume, was washed in 50 ml 0.1M sodium acetate containing 5 mM sodium periodate, pH 5, in a glass, stoppered tube. Following end-over-end mixing for 1 hour at room temperature 5 ml 10% (v/v) glycerol was added to utilise the excess periodate and the slurry washed for a further 1 hour. The gel was then washed with 2 l 0.1M sodium bicarbonate pH 9.

Coupling reaction with sheep anti-mouse-immunoglobulin immunoglobulin G (IgG)

Oxidised Sephacryl S300, 15 ml settled volume, was resuspended in 20 ml of a protein solution (18% sodium sulphate IgG fraction from sheep serum), protein concentration between 5 and 8 mg/ml. The suspension was left, end-over-end mixing, at room temperature for between 16 and 18 hours. The uncoupled protein was removed by filtration.

Reduction with sodium borohydride

The coupled solid phase antibody was resuspended in 50 mM phosphate buffered saline, pH 7.5, allowed to settle for 30 minutes and the supernatant removed. The solid phase antibody was then resuspended in phosphate buffered saline to a final volume of 100 ml and 500 mg sodium borohydride added, with stirring. The slurry was left, with intermittent stirring, for 30 minutes at room temperature.

Following reduction the solid phase antibody was washed twice with phosphate buffered saline then 5 times with 0.25M Tris HCl, pH 8.5, containing 1% Tween 20 and 2% horse serum. The settled gel, 15 ml, was resuspended in 20 ml 0.25M Tris HCl, pH 8.5, containing 1% Tween 20, 2% horse serum and 0.01% sodium azide and stored at $4^{o}C$ until use.

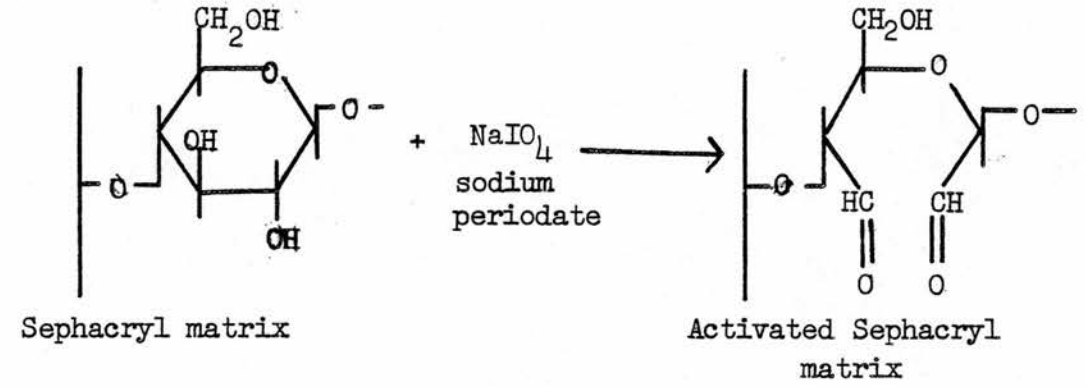
### 8.1.4 Assay for anti-GAD activity

A two step assay method to detect anti-GAD antibodies was developed. The GAD activity in cytosol obtained from whole chick brain was removed by double antibody precipitation using solid phase anti-mouse antibody. Any residual GAD activity was detected using a radiometric GAD assay essentially as described in Section 4.1.2.

Preparation of chick brain cytosol

One three week old chick was decapitated, the brain rapidly removed and homogenised, 1:3 (w/v) in ice cold 100 mM potassium phosphate buffer containing 1 mM AET and 0.1 mM PLP, pH 7.2. The homogenate was centrifuged at 100 000 $g_{av}$, $4^{o}C$ for 1 hour and the resulting cytosol used as a source of GAD activity.

Activation with sodium periodate




Addition of ligand

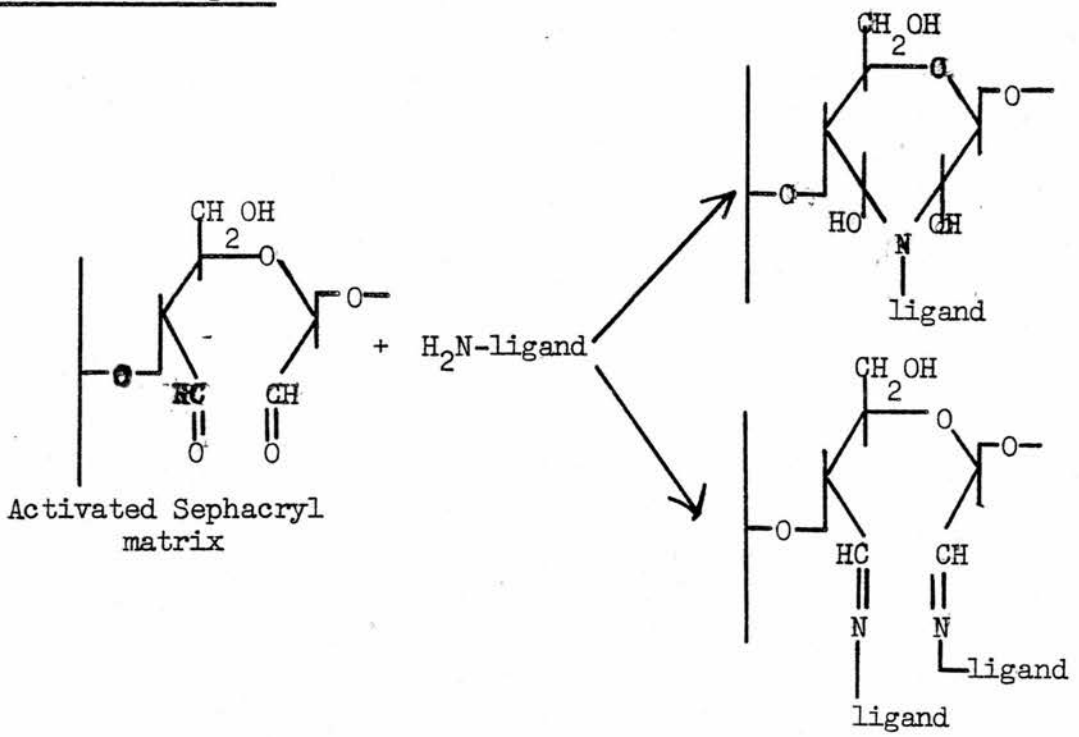



Figure 8.1 Schematic diagram showing the manner in which sheep anti-mouse-immunoglobulin G was coupled to Sephacryl S300. See text section 8.1.3. for details.

Double antibody precipitation

Equal volumes (20 µl) of cytosol and the sample to be tested (mouse antiserum, medium taken from wells or flasks containing hybrid cells, ascites fluid) were mixed in an Eppendorf pot containing 5 µl foetal calf serum. After 30 or 45 minutes at room temperature 20 µl of well mixed, solid phase anti-mouse antibody was added (see Section 8.1.3). The samples were left at room temperature for a further 30 or 45 minutes with gentle intermittent shaking. (Care must be taken to ensure that, whilst shaking is not vigorous or prolonged enough to cause "frothing" which inactivates the enzyme, the beads are kept evenly dispersed in the protein solution.) The samples were then centrifuged for 10 seconds using a Beckman microfuge and 5 µl samples withdrawn from the supernatant for assay (in triplicate) for GAD activity.

The controls used for the assay differed slightly depending on the source of the sample assayed and will be noted in the experimental procedure for the individual experiments.

In early experiments problems were encountered due to protease activity in the chick brain cytosol during incubation at room temperature which led to highly variable results. The most effective method of counteracting this activity was to add a small amount of foetal calf serum which contains several protease inhibitors (see Discussion).

8.1.5 Production of monoclonal antibodies to GAD

8.1.5.1 Tumour cell line

The parent tumour cell line used was a Balb/c tumour cell line X6 3NS-1. This cell line lacks the enzyme hypoxanthine (guanine) phosphoribosyl transferase (H(G)PRTase EC.2.4.2.8) and therefore cannot survive in HAT (hypoxanthine, aminopterin, thymidine) selective medium (Littlefield 1964).

8.1.5.2 Media

The parental cell line was maintained in stationary suspension cultures (cell concentration between $10^5$ and $10^6$/ml) in NS-1 medium which is RPMI-1640 medium containing 2 mM L-glutamine, 1 mM sodium pyruvate (sodium pyruvate MEM), 10% foetal calf serum and antibiotics, 500 units streptomycin and 500 units penicillin per 500 ml medium.

The fusion of spleen cells with X6 3NS-1 cells was carried out in serum free NS-1 medium.

HAT medium is NS-1 medium containing 2% (v/v) of a HAT stock solution. HAT stock solution consists of 100 ml of a solution containing 38.8 mg thymidine and 136.1 mg hypoxanthine and 10 ml of a solution containing 1.76 mg aminopterin made up to 2oo ml with double distilled water.

HT medium is NS-1 medium containing 2% (v/v) of a HT stock solution. The HT stock solution is HAT stock solution with aminopterin omitted.

All media were sterilised by membrane filtration.

The 35% (w/v) polyethylene glycol solution, $M_r$ approximately 1500 (PEG 1500) was made up fresh each time by adding 0.7 g PEG (sterilised by autoclaving for 10 minutes at 15 lb/sq. in) to 1.3 ml serum free NS-1 medium containing 10 ul sterile 2M Tris base.

8.1.5.3 Immunisation of mice

Balb/c mice were injected intraperitoneally with 200 µl of a mixture of partially purified GAD (see Section 5.1) and Freund's complete adjuvant, 1:1 (v/v). Prior to mixing with adjuvant the GAD solution was concentrated (see Section 3.3.2) so that the animals each received approximately 100 µg protein. This primary injection was followed, 3 weeks later, by a second intraperitoneal injection with 100 µl (approximately 100 µg) partially purified GAD. The mice were then divided into 2 groups A and B.

Group A

Three weeks after the last injection each mouse was injected (subcutaneously into several sites) with 300 µl partially purified GAD (approximately 300 µg protein) and 50 µl Freund's complete adjuvant. After another interval of 3 weeks each mouse was injected intravenously with 100 µl partially purified GAD and on the 4th day after injection the spleen was removed from 1 mouse for fusion, Fusion 1.

Group B

Four weeks after the last injection each mouse was injected (subcutaneously into several sites) with 250 µl (approximately 250 µg protein) partially purified GAD and 50 ul Freund's complete adjuvant. This was followed, at intervals of 3 weeks, by 2 more injections of 100 µl of partially purified GAD, subcutaneously into several sites. After a further 3 weeks the mice were injected intravenously with 200 µl partially purified GAD and on the 4th day after injection the spleen was removed from 1 mouse for the fusion, Fusion 2.

8.1.5.4 Fusion of spleen cells with X63NS-1 cells

This was carried out by an adaptation of the method used by Kennet et al (1978).

The spleen was removed aseptically and homogenised gently to give a cell suspension which was washed twice in serum free NS-1 medium. After the second wash the supernatant was removed and 5 ml of ice cold sterile ammonium chloride (0.17M) was added to lyse red blood cells. Ten minutes later serum free NS-1 was added to a final volume of 25 ml and the cells pelleted by centrifugation (1000 rpm for 5 minutes). After a further wash with serum free NS-1 the cells were counted and the appropriate number of X63NS-1 cells (which had also been washed twice in serum free NS-1 medium) added. Approximately $10^7$ X63NS-1 cells were added to between 5 and 10 x $10^7$ spleen cells. The volume was made up to 25 ml with serum free NS-1 and the cells pelleted by centrifugation (1200 rpm for 5 minutes).

All the supernatant was removed, the pellet tapped loose and 0.5 ml 35% (w/v) PEG 1500 added and the cells resuspended by swirling gently. The cell suspension was centrifuged at 700 rpm for between 3 and 5 minutes then 8 minutes after the addition of the PEG 5 ml serum free NS-1 medium was added, over a period of approximately 2 minutes without disturbing the pellet. The cells were resuspended, over a period of between 3 and 4 minutes, by swirling gently so as not to dislodge the whole pellet at once. After centrifugation at 1200 rpm for 5 minutes the supernatant was removed and 5 ml HAT medium added slowly so that the pellet was not disturbed. After 5 to 7 minutes the pellet was resuspended gently, the volume made up so that there were 2 x $10^6$ cells/ml and 100 $\mu$l aliquots added to each well of 4 Sterilin (96 well) flat bottom tissue culture plates, onto a macrophage feeder layer. The feeder cells were freshly harvested peritoneal macrophages from Balb/c mice which had been plated at $10^4$ cells/well in HAT medium the previous day.

The cells were maintained in HAT medium for 14 days and then this was replaced with HT medium. After the clones had appeared macroscopically samples of the medium were removed for assay for antibodies to GAD (see Section 8.1.4). Since the medium had been changed at least twice before samples were taken no residual antibody, secreted by unfused spleen cells, remained to give falsely positive results. After a change of medium a minimum of 4 days elapsed before medium was removed for assay.

Any hybrids that secreted antibodies which affected GAD activity were expanded into 24 well flat bottom tissue culture plates and subsequently into small, flat bottomed tissue culture flasks holding approximately 5 ml medium. Cells from one of the hybrids produced from Fusion 2 (approximately $10^7$ cells in a volume of 0.5 ml) were injected intraperitoneally into 7 Balb/c mice 7 days after a primary injection of pristane. The ascites fluid which was produced was screened for anti-GAD activity as described in Section 8.1.4.

## 8.2 Experimental

### 8.2.1 Demonstration of anti-GAD activity in mouse serum

#### 8.2.1.1 Inhibition of GAD activity by serum from mice immunised against GAD

Three Balb/c mice were injected intraperitoneally with 250 µl of a mixture of partially purified GAD, approximately 125 µg total protein, and Freund's complete adjuvant. Eight days later each mouse received 50 µl partially purified GAD intravenously. Nine days later blood samples were taken from blood vessels near the eye, the blood allowed to clot and the serum removed after centrifugation for 5 minutes in a Beckman microfuge.

Equal volumes (15 µl) of chick brain cytosol, prepared as described in Section 8.1.4, and each of the mouse sera were mixed then left on ice overnight (approximately 16 hours). The samples were centrifuged for 5 minutes in a Beckman microfuge, and 5 µl samples of the supernatants removed for assay for GAD activity as described in Section 4.1.2. Each GAD assay was performed in triplicate. Controls consisted of replacing serum taken from mice which had been immunised against partially purified GAD, ie "immune" serum, with serum from mice which had not been immunised against GAD, ie "non immune" serum, or with 100 mM potassium phosphate buffer, pH 7.2, containing 1 mM AET and 0.1 mM PLP.

#### 8.2.1.2 Precipitation of GAD activity by a double antibody method

The three mice sera used were the same as those described in the previous experiment. Chick brain cytosol was prepared as described in Section 8.1.4.

Four slightly different sets of experimental conditions were used:

| | | | | | | |
|---|---|---|---|---|---|---|
| A. | 15 µl mouse serum + 15 µl cytosol | 25°C / 1 hour | 15 µl sheep anti-mouse antibody | ice / 2 hours | centrifuge for 5 minutes in microfuge |
| B. | 15 µl mouse serum + 15 µl cytosol | ice / 3 hours | 15 µl sheep anti-mouse antibody | ice / 2 hours | centrifuge for 5 minutes in microfuge |
| C. | 15 µl mouse serum + 15 µl cytosol | 25°C / 1 hour | 15 µl sheep anti-mouse antibody | ice / overnight | centrifuge for 5 minutes in microfuge |
| D. | 15 µl mouse serum + 15 µl cytosol | ice / 3 hours | 15 µl sheep anti-mouse antibody | ice / overnight | centrifuge for 5 minutes in microfuge |

Controls consisted of replacing "immune" serum with "non immune" serum or potassium phosphate buffer for each set of experimental conditions. The sheep antiserum to mouse immunoglobulins was added to precipitate any mouse immunoglobulins in the mouse serum.

After centrifugation 5 µl samples of the supernatants were removed for assay for GAD activity as described in Section 4.1.2. In addition the pellets from A and B were resuspended in standard buffer and assayed for GAD activity. Each assay was performed in triplicate.

8.2.1.3 Precipitation of GAD activity by a double antibody method using solid phase anti-mouse antibody

Blood samples were taken from a mouse in group A (see Section 8.1.5.3) 10 days after the last intravenous injection. Anti-GAD activity in the serum was detected as described in Section 8.1.4 however, the incubation conditions were varied slightly. Incubation A was exactly as described in Section 8.1.4. Incubation B, 20 µl of a 1/100 dilution of mouse serum in phosphate buffer was added to the incubation. Incubation C, 20 µl of a 1/4 dilution of solid phase antibody was added to the incubation. Controls consisted of replacing "immune" serum with "non immune" serum or phosphate buffer.

8.2.2 Screening for production of monoclonal antibodies against GAD

Samples of medium were taken from wells or flasks containing hybrid cells and assayed for anti-GAD activity as described in Section 8.1.4. When very large numbers of samples were involved, equal volumes of medium, taken from several wells, were combined and a sample of the mixture assayed. If anti-GAD activity was detected in any of these "pools", the individual samples were assayed. Controls included samples of medium from wells which contained hybrids from an unrelated fusion. Any positive wells were retested several times, usually as blind tests, included amongst other new samples or media samples taken from unrelated fusions.

Double immunodiffusion was carried out as described in Section 8.1.1, with samples from those wells containing hybrids and sheep anti-mouse-immunoglobulin antiserum to detect immunoglobulins.

Ascites fluid samples taken from mice which had been injected with hybridoma cells from a positive well were assayed for anti-GAD activity as described in Section 8.1.4. Controls included ascites fluid from mice injected with hybridoma cells from an unrelated fusion.

## 8.3 Results

### 8.3.1 Demonstration of anti-GAD activity in mouse serum

#### 8.3.1.1 Inhibition of GAD activity by serum from mice immunised against GAD

Table 8.1 shows the effects on GAD activity when chick brain cytosol was mixed with serum taken from mice immunised against GAD. Section 8.2.1.1 contains experimental details.

The results shown in Block A indicated that serum from mice 1 and 2 contained anti-GAD antibodies whereas serum from mouse 3 contained little or no anti-GAD activity. However, when the experiment was repeated exactly, Block B, only the serum from mouse 2 showed any anti-GAD activity.

The variability in results which occurred when the experiment was performed in this manner could be attributed to the presence of soluble antigen-antibody complexes which had retained enzyme activity. Although the mouse serum contained anti-GAD activity the mice were not highly immune since a 10 fold dilution of the serum was sufficient to reduce anti-GAD activity to an undetectable level. Consequently it is possible that these incubations were approaching antigen excess so that there was incomplete lattice formation and therefore a different proportion of the GAD activity was precipitated when the experiment was repeated.

The results might be interpreted as follows. The serum from mouse 1 and 2 contained anti-GAD antibodies, although the serum from mouse 2 may have had a higher titre since the effect proved to be reproducible whereas mouse 1 may have had a lower titre and in the second experiment no antigen-antibody complexes precipitated due to antigen excess. This could have been caused by using chick brain cytosol which had a slightly higher protein concentration. Serum from mouse 3 seemed to contain little or no anti-GAD activity.

Double immunodiffusion of samples of serum from the three mice versus partially purified GAD did not result in the formation of precipitin bands. However, it is possible that this might be the result of using an unconcentrated sample of antigen (partially purified GAD from G200 peak fractions). Consequently the amount of protein in the antigen wells, 1 µg or less, would be insufficient for GAD to be detected by this technique.

Table 8.1. Inhibition of GAD activity by serum taken from mice immunised against GAD

| | A | | B | |
|---|---|---|---|---|
| GAD activity | % buffer control | % non immune serum control | % buffer control | % non immune serum control |
| Mouse 1 | 58 | 62 | 86 | 96 |
| Mouse 2 | 51 | 54 | 53 | 59 |
| Mouse 3 | 85 | 90 | 91 | 100 |

The table shows the GAD activity which remained after chick brain cytosol was mixed with serum taken from 3 mice. Experimental conditions are described in Section 8.2.1.1. Block A and Block B represent identical experiments. GAD activity is expressed as a percent of that in the buffer control and as a percent of the GAD activity in the non immune serum control.

8.3.1.2 Precipitation of GAD activity by a double antibody method

Table 8.2 shows the GAD activity which remained in chick brain cytosol after double antibody precipitation using serum from mice immunised against GAD and sheep anti-mouse-immunoglobulin antibody. The GAD activity in the pellets, which were resuspended in standard buffer, was also determined. A, B, C and D represent four slightly different experimental conditions with regard to temperature and the length of time allowed for antigen-antibody complexes to form and to precipitate. Experimental details are contained in Section 8.2.1.2.

There was no marked difference in the percentage activity remaining in the supernatant whether the GAD-anti GAD complex was allowed to form at 25°C for 1 hour or on ice for 3 hours, columns A and B, Table 8.2. Similarly, there was no marked difference in the results whether the anti-GAD-anti-mouse-immunoglobulin complex was left to form for 2 hours or overnight, columns C and D.

The results again indicate that serum from mouse 2 contains the highest anti-GAD activity. However, in this experiment the sera from mice 1 and 3 seem to contain similar amounts of anti-GAD activity whereas in the previous experiment serum from mouse 3 did not appear to contain any anti-GAD activity.

A possible reason, as suggested for the previous experiment, might be antigen excess which prevented lattice formation and hence precipitation in the absence of a second antibody. The antigen:antibody ratio could have been greater when serum from mouse 3 was used than when serum from mouse 1 was used. Consequently there was no indication of any anti-GAD activity. In the presence of a large excess of the second antibody any mouse immunoglobulin would be precipitated so that the effects of only a small concentration of the first antibody would be detected.

Whereas, for mouse 2 when the percent GAD activity which remained in the supernatant was added to the percent GAD activity in the pellet, the value was approximately 100%, this did not prove to be the case for mice 1 and 3. A possible reason might be different antibody specificities, both of the first and second antibody. Data from this and the previous experiment suggest that although there are anti-GAD antibodies in the serum from mice 1 and 3, interaction with GAD does not prevent enzyme activity. Different combinations of antibody specificity however, might result in the blocking of enzyme activity upon addition of the second antibody (sheep anti-mouse-immunoglobulin antibody) in the case of serum from mice 1 and 3 but not with serum from mouse 2.

Table 8.2. Precipitation of GAD by a double antibody method

| | % GAD activity | | | |
|---|---|---|---|---|
| | A | B | C | D |
| Mouse 1 | 28 (33) | 36 (13) | 32 | 33 |
| Mouse 2 | 18 (81) | 19 (88) | ND | ND |
| Mouse 3 | 43 (17) | 36 (5) | 25 | 33 |

The table shows GAD activity which was not precipitated from chick brain cytosol. Experimental conditions are described in Section 8.2.1.2. GAD activity is expressed as a percent of that in the buffer and non immune serum controls. Figures in brackets represent the GAD activity in the pellets expressed as a percentage of the controls. ND = not determined.

The results described in these two experiments, in addition to the fact that it only proved possible to demonstrate anti-GAD activity using undiluted serum, suggested that although injection of partially purified GAD had provoked an immune response, the titre of anti-GAD antibodies was very low indeed.

8.3.1.3 Precipitation of GAD activity by a double antibody method using solid phase anti-mouse antibody

Table 8.3 shows the effects of reducing the concentration of mouse serum and the solid phase anti-mouse antibody. When the mouse serum was diluted 100 fold, the amount of GAD precipitated was reduced as shown by a 29% increase in the enzyme activity which remained in the supernatant. This demonstrated that the amount of solid phase antibody added was adequate to precipitate all the mouse immunoglobulin present in this sample of antiserum. If the amount of solid phase antibody added had been inadequate, a dilution of mouse serum would have resulted in increased precipitation of GAD since all, rather than a percentage, of the mouse immunoglobulins would be precipitated. Similarly, the four fold dilution of solid phase antibody would have resulted in a much higher percentage of unprecipitated GAD. However, anti-GAD activity was still demonstrable using a 100 fold dilution of mouse serum which indicates that the antibody concentration was higher than previously.

8.3.2 Screening for production of monoclonal antibodies against GAD

8.3.2.1 Fusion 1

From this fusion, using the spleen from a mouse in group A, 3% of the wells seeded contained hybrids. Samples of medium taken from these wells were tested for anti-GAD activity. In addition double immunodiffusion versus sheep anti-mouse-immunoglobulin antibody was carried out to determine the presence of immunoglobulins in the medium (see Section 8.2.2). The results are shown in Table 8.4. The hybrids were screened again for anti-GAD activity 5 days after the medium had been changed with very similar results.

The medium from well 12 apparently contained quite a high concentration of anti-GAD activity since a 100 fold dilution of the medium more than doubled the amount of precipitation. In addition there was a very strong precipitin band produced by double immunodiffusion indicating a high immunoglobulin concentration. The cultures were expanded into 24 well flat bottom tissue culture plates however, during this procedure the cultures became contaminated (either bacterial or fungal contamination) and the cells were lost.

Table 8.3. Precipitation of GAD by a double antibody method using solid phase anti-mouse antibody

| | | % GAD activity remaining in supernatant |
|---|---|---|
| A | Undiluted serum and undiluted solid phase | 35 |
| B | 1/100 serum and undiluted solid phase | 64 |
| C | Undiluted serum and 1/4 solid phase | 42 |

The table shows the effect on the precipitation of GAD when the concentration of mouse serum or solid phase anti-mouse antibody was varied. The experimental conditions are described in Section 8.2.1.3. GAD activity is expressed as a percentage of the activity in the controls.

Table 8.4. Screening of hybrids from fusion 1 for anti-GAD activity

| Well no. | % GAD activity | | Precipitin arc after immunodiffusion |
|---|---|---|---|
| | Undiluted | 1/100 dilution | |
| 1 | 78 | 95 | Tr |
| 2 | 91 | 73 | - |
| 3 | 100 | 70 | - |
| 4 | 71 | 91 | Tr |
| 5 | 69 | 76 | - |
| 6 | 90 | 71 | - |
| 7 | 80 | 67 | ++ |
| 8 | 100 | 87 | - |
| 9 | 80 | 84 | - |
| 10 | 76 | 84 | ++ |
| 11 | 61 | 81 | - |
| 12 | 64 | 30 | +++ |

Samples of media taken from each well containing hybrids was assayed for anti-GAD activity as described in Section 8.1.4. Double immunodiffusion was carried out by the Ouchterlony method as described in Section 8.1.1. GAD activity is expressed as a percentage of the control values. Each assay was performed in triplicate.
Tr = trace

8.3.2.2 Fusion 2

From this fusion, using the spleen from a mouse in group B, 20% of the wells seeded contained hybrids. Samples of medium taken from these wells were tested for anti-GAD activity. Table 8.5 shows the results of the initial tests. Since, in the first instance, a large number of samples were involved several were combined together for assay as described in Section 8.2.2.. When samples of medium from the positive "pools" (those where enzyme activity was reduced to 70% or less) of wells 1 to 58 were assayed individually the medium from well 54 contained the most anti-GAD activity, reducing enzyme activity to 52%, followed by well 45 at 59% and well 42 at 69%.

At this stage the cultures were expanded into 24 well tissue culture plates during which contamination occurred resulting in the loss of many of the hybrids including number 54. Hybrids 59 to 77 were lost before they could be retested. Of those positives which had been tested individually only 42 and 45 remained. Since there was no opportunity to test the majority of the positive hybrids by diluting the medium before determining anti-GAD activity it is not known whether hybrid 54 had secreted the most anti-GAD activity or whether one of the apparently less positive hybrids would prove to have secreted more anti-GAD antibody.

Table 8.6 shows the results when double immunodiffusion was carried out with samples of medium from each well and sheep anti-mouse-immunoglobulin antibody. Control wells contained mouse serum taken from a mouse which was hyperimmune to an unrelated antigen. It is interesting that with expansion into larger wells hybrids 42 and 45 secreted higher concentrations of immunoglobulin than previously. This coincides with an increase in anti-GAD activity (see Figure 8.2).

Figure 8.2 shows the effects on the amount of anti-GAD activity present in the medium as hybrids 42 and 45 were expanded from 96 well to 24 well tissue culture plates and finally to small, flat bottomed tissue culture flasks which hold approximately 5 ml medium. Although the results are not shown test number 3 and any subsequent tests were also carried out using a 100 fold dilution of medium. The reduction in GAD activity obtained was not markedly different from that obtained using undiluted medium.

Table 8.5. Screening of hybrids from fusion 2 for anti-GAD activity

| Well numbers | % GAD activity | Well numbers | % GAD activity |
|---|---|---|---|
| 1+ 2+ 3 | 90 | 49+50+51 | 66 |
| 4+ 5+ 6 | 77 | 52+53+54 | 34 |
| 7+ 8+ 9 | 81 | 55+56+57 | 70 |
| 10+11+12 | 89 | 58 | 100 |
| 13+14+15 | 100 | 59+50 | 89 |
| 16+17+18 | 94 | 61+62 | 62 |
| 19+20+21 | 82 | 63+64 | 63 |
| 22+23+24 | 81 | 65+66 | 80 |
| 25+26+27 | 90 | 67+68 | 77 |
| 28+29+30 | 69 | 69+70 | 100 |
| 31+32+33 | 69 | 71+72 | 98 |
| 34+35+36 | 71 | 73+74 | 73 |
| 37+38+39 | 91 | 75 | 71 |
| 40+41+42 | 62 | 76 | 84 |
| 43+44+45 | 60 | 77 | 52 |
| 46+47+48 | 66 | | |

Equal volumes of medium from each well was combined as shown in the table and assayed for anti-GAD activity as described in Section 8.1.4. GAD activity is expressed as a percentage of the control values. Each assay was performed in triplicate.

Table 8.6. Double immunodiffusion of media samples from wells containing hybrids versus sheep anti-mouse antibody

| Well no. | Anti-Ig | 1/10 Anti-Ig |
|---|---|---|
| 15 | + | + |
| 16 | + | + |
| 19 | Tr | + |
| 30 | - | + |
| 35 | Tr | + |
| 42 | - | Tr |
| 45 | - | + |
| 54 | + | ++ |
| 62 | tr/+ | + |
| 69 | + | ++ |
| 72 | tr/+ | + |
| 42* | ++ | ++/+++ |
| 45* | ++ | ++/+++ |
| Mouse serum | +++ | ++ |
| 1/10 mouse serum | ++ | +++ |

Double immunodiffusion of samples of media from wells containing hybrids versus sheep anti-mouse-immunoglobulin antibody and versus a 1/10 dilution of sheep anti-mouse-immunoglobulin antibody. Samples from all the wells were assayed but only those where precipitation occurred have been included. * indicates samples taken at a later stage of the procedure after the cultures had been expanded to 24 well plates.

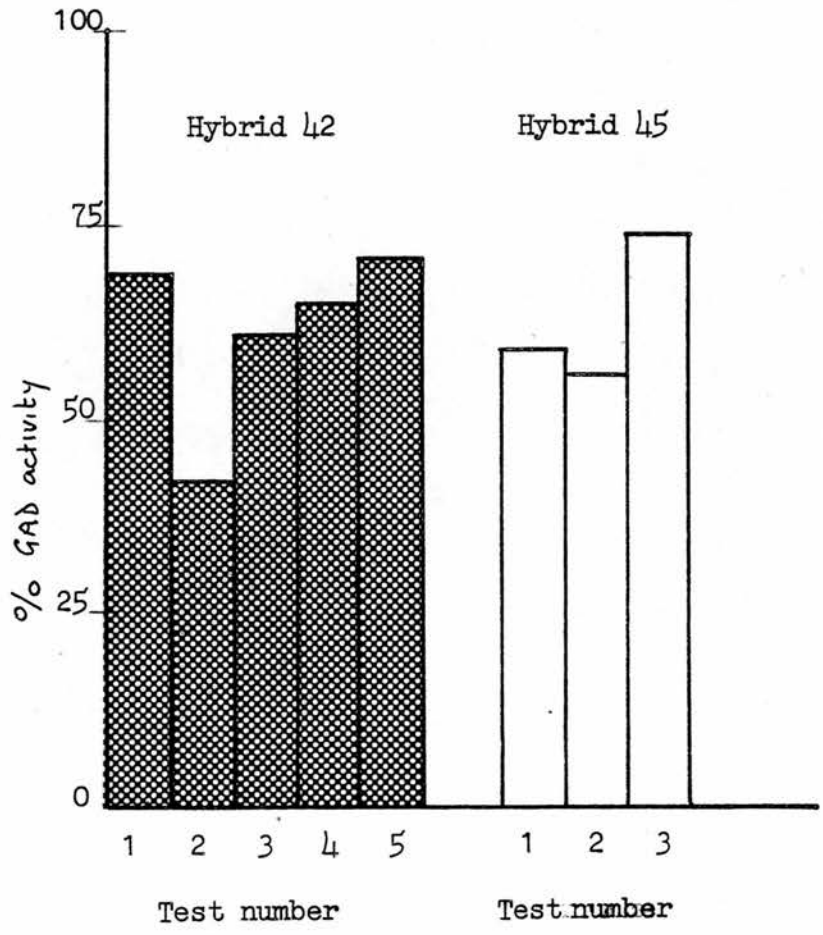



Figure 8.2 The effects on anti-GAD activity as cultures were expanded from 96 well tissue culture plates to 5 ml tissue culture flasks. Tests 1 to 5 were carried out in chronological order. Test 1, medium from 96 well tissue culture plates; Test 2, medium from 24 well tissue culture plate; test 3, medium from 24 well tissue culture plate; Tests 4 and 5, successive tests on medium from 5 ml flask.
See section 8.2.2. for experimental details. GAD activity is expressed as a percentage of the control values.

Table 8.7. Inhibition of GAD activity by ascites fluid from mice infected with hybridoma cells

| | % GAD activity remaining | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Day no. | Mouse 1 | | Mouse 2 | | | | Mouse 3 | | | Mouse 4 | Mouse 5 |
| | Undil. | 1/50 | Undil. | 1/10 | 1/50 | 1/100 | Undil. | 1/10 | 1/50 | Undil. | Undil. |
| 1 | | | 61 | 79 | 63 | 58 | | | | 67 | 72 |
| 2 | 63 | 90 | - | 73 | 68 | - | - | 71 | 65 | | |
| 3 | | | | | | | | | | 69 | 100 |
| 4 | 73 | 74 | - | 73 | 69 | - | - | 65 | 60 | | 72 |
| 5 | 72 | 87 | - | 82 | 97 | - | - | 100 | 62 | 59 | 64 |
| 6 | 76 | - | 100 | - | - | - | 69 | - | - | | 89 |
| 7 | | | | | | | 74 | - | - | | 73 |
| 8 | | | | | | | | | | | 55 |

The table shows the GAD activity remaining after precipitation by a double antibody method with ascites fluid at the dilutions shown, and solid phase anti-mouse antibody as described in Section 8.1.4. GAD activity is expressed as a percentage of the controls. See Section 8.2.2. - = not determined.

There was an initial increase in anti-GAD activity immediately after the transfer to the 24 well tissue culture plate however, as the hybrids grew, anti-GAD activity declined. Whereas hybrid 42 was expanded into flasks, the cells of hybrid 45 stopped dividing before there was a sufficient number to transfer to flasks. From Figure 8.2 it would appear that both hybrids were losing their ability to secrete anti-GAD antibody. However, once antibody forming hybrids have been identified it is not unusual for them to lose the ability to secrete antibody as they are grown up (Yeldon et al 1978). This could be due to chromosome loss at cell division (Margulies et al 1976; Milstein et al 1976; Kohler 1980) which might also provide an explanation for the loss of the ability to divide of the cells of hybrid 45. An alternative possibility is that if the hybrid producing anti-GAD antibody is one of a number of hybrids in a given well it may be overgrown by the others.

Cells from hybrid 42 were injected intraperitoneally into 7 mice and samples of ascites fluid taken over an 8 day period. Two of the mice died after only two samples had been taken from each. When these undiluted samples were tested for anti-GAD activity enzyme activity was reduced by 26% and 24%, 0% and 22%. Table 8.7 shows the results when ascites fluid samples from the other 5 mice, taken on the days shown, were assayed for anti-GAD activity. A number of the samples were assayed at more than one dilution and ideally all the samples should have been assayed over a range of dilutions to ensure the optimum conditions for complete precipitation. However, this proved to be impractical. In those cases where more than one dilution of the ascites fluid was assayed, the dilution did not result in a marked increase in the precipitation of GAD activity.

The results indicate that the ascites fluid from some of the mice contained anti-GAD antibodies (mice 5, 4 and 3 seemed to have the best response) however, the concentration did not appear to be particularly high.

## 8.4 Discussion

Initially it had been intended to raise antibodies to pure chick brain GAD in the conventional manner by immunising rabbits and using the serum as a source of anti-GAD antibodies. Monoclonal antibodies however, eliminate the necessity of using a pure protein as immunogen since cell lines synthesising monoclonal antibodies may be derived using an immunogen which is not pure. The multiple components of the heterogeneous cell population which will be produced by the fusion between the spleen and myeloma cells can be resolved by cloning.

A major problem proved to be producing immunity in the mice. Although repeated injections with partially purified GAD did result in anti-GAD activity in the serum, the antibody titre was obviously quite low (see Table 8.3). This suggests that GAD which is a soluble protein located mainly in the cytosol is not particularly immunogenic. Under certain conditions GAD does bind to membranes (see Introduction) but the purification procedure used selected for soluble rather than membrane bound GAD. The mice which had demonstrated the highest degree of immunity, on the basis of the assay for anti-GAD activity, were selected for each fusion.

Antibodies to mouse, rat, catfish and bovine brain GAD (Saito et al 1974; Maitre et al 1978(b); Su et al 1979; Wu 1982) have been raised by immunising rabbits with the purified protein. There were no reports of any difficulties in causing hyperimmunity in rabbits which may perhaps be attributed to differences in immune responsiveness between rabbits and mice.

During the initial stages of the development of an assay for anti-GAD activity the assays were left overnight on ice for the immune complexes to form (Saito et al 1974) however, as can be seen from Table 8.2 this proved to be unnecessary.

Since the primary antigen-antibody complex is established within seconds (Tengerdy & Small 1966) and, at room temperature, the final polymerised antigen-antibody complex forms within about 30 minutes (Hughes-Jones et al 1963) the incubation conditions were revised (see Section 8.1.4). However, the increase in temperature resulted in marked, unspecific losses in enzyme activity which were attributed to protease activity. When the whole procedure had been carried out on ice any protease activity had been minimal and as a consequence

overlooked. The addition of a small amount of foetal calf serum prevented this unspecific loss of enzyme activity. Serum contains a number of protease inhibitors many of which act on serine proteases which are the most active proteases at the pH and under the buffer conditions used in this study (Barret 1977). Although a number of the protease inhibitors in serum are quite specific in their action, several have quite a wide range of action and one in particular $\alpha$-2 macroglobulin will inhibit the majority of proteases (Starkey & Barret 1977).

Although the first fusion (Table 8.4) was not particularly successful as regards numbers of hybrids, one of those produced did appear to secrete quite a high concentration of anti-GAD antibody. When undiluted medium from the well was tested the GAD activity in chick brain cytosol was reduced to 64%. When the medium was diluted 100 fold, GAD activity was reduced to 30%. When undiluted medium was used the second antibody was not sufficient to precipitate all the immunoglobulin secreted by the cells. When the medium was diluted a higher percentage of the antibody present was precipitated.

Double immunodiffusion experiments confirmed that there was a high concentration of immunoglobulins present. This is not a particularly sensitive method of determining the presence of immunoglobulin since lack of precipitation does not necessarily mean that the cells are not secreting immunoglobulin, just that the level is too low for precipitation to occur or to be detectable. However, more sensitive methods such as radioimmunoassay or enzyme linked immunoassay require pure antigen or pure antibody.

Fusion 2 (see Table 8.5) resulted in a much higher percentage of hybrids than Fusion 1, a number of which seemed to contain anti-GAD activity. Unfortunately, due to contamination a large number of the hybrids were lost and only two of those which appeared to secrete anti-GAD activity remained. As these two hybrids were expanded the rate of cell division of hybrid 45 showed down and eventually cell division ceased. This was accompanied by a decrease in the ability to produce antibodies. It was suggested that the loss of the ability to divide might have been caused by chromosome loss at cell division.

There are a number of factors which might contribute to the loss of the ability to secrete antibodies by hybrids apart from those already mentioned, namely chromosome loss or overgrowth by other more rapidly

dividing cells. It is possible that as the cells divide the hybridomas might generate variants whose affinity might have changed and which might overgrow the original hybrids (Cook & Scharff 1977).

Even if the hybrid has been cloned there may be sudden, gradual loss of the ability to produce antibody presumably due to chromosome loss (Yeldon et al 1978). In addition subclones of apparently stable hybrid cell lines were isolated which produced only 50% of the antibody and overgrew the other cells which eventually died.

It is interesting that there was a considerable increase in the concentration of immunoglobulins in the medium after hybrids 42 and 45 had been expanded into 24 well tissue culture plates. This coincided, particularly in the case of hybrid 42, with an increase in anti-GAD activity. However, anti-GAD activity subsequently started to decline in both well 42 and 45. At this stage hybrid 45 ceased to grow and hybrid 42 was expanded into flasks where the gradual decrease in anti-GAD activity in the medium continued, although the immunoglobulin concentration remained high. This might indeed be indicative of overgrowth of the hybrid secreting anti-GAD activity by other immunoglobulin secreting cells. In retrospect greater success might have been achieved if the cultures had not been expanded to the same extent but had been cloned at an early stage.

Cells from hybrid 42 were injected into mice in an attempt to "boost" the anti-GAD activity. Any ascites tumours formed from hybrid cells secreting anti-GAD antibody would in turn secrete large amounts of the homogeneous antibody. Although this would be contaminated by immunoglobulins from ascites tumours formed from other antibody secreting cells and to a certain extent by the immunoglobulin repertoire of the recipient mouse, in the event of a good response in terms of anti-GAD activity, the antibody, although not homogeneous might have proved useful. For example in the preparation of an immunoadsorption column for purification of GAD or in an attempt to identify which of the protein bands in a polyacrylamide gel represented GAD.

## SECTION 9: REGIONAL VARIATION IN GABA METABOLISM IN CHICK BRAIN

### 9.1 Methods

#### 9.1.1 Histological techniques

##### 9.1.1.1 Preparation of frozen sections

Whole chick brains were placed on microtome chucks, covered with embedding medium (Tissue Tek II O.C.T. compound) and quenched in liquid nitrogen. The chucks were kept, usually overnight, at -20°C before the tissue was sectioned to allow temperature equilibration. Transverse frozen sections (20 μm) were cut in a Pearse-Slee cryostat at -20°C, thawed onto glass slides and allowed to dry, at 4°C if the sections were to be used for histochemistry, otherwise under a stream of warm air.

##### 9.1.1.2 Staining sections

Tissue sections were stained with cresyl violet for examination of tissue morphology. Cresyl violet binds to RNA and stains the cell bodies of neurons (nucleus and cytoplasm which is rich in RNA-containing Nissl granules) and to a lesser extent glial cells (nuclei only).

Cresyl violet (0.1 g) was dissolved in 10 ml absolute ethanol, made up to 75 ml with double distilled water and stirred for 24 hours. A 9 ml aliquot of this stock solution was added to 100 ml 0.1M acetic acid which had been corrected to pH 4.5 with NaOH.

The slides were immersed in stain for 30 minutes, washed quickly in distilled water and the sections dehydrated in alcohol by immersing for 10 seconds in 75%, 95% and absolute ethanol. After clearing in xylene the sections were mounted in DEPEX mounting medium. The sections were examined using a Vickers M17 fluorescence microscope fitted with a 35 mm camera and automatic exposure meter. Photographs were taken using Ilford Pan F ASA 50 black and white film.

##### 9.1.1.3 Histochemical localisation of GABA-T

The histochemical technique used to demonstrate GABA-T activity was that described by Hyde and Robinson (1976).

###### 9.1.1.3.1 Perfusion fixation of chick brain

Chick brain tissue was fixed by vascular perfusion with a mixture of 2% formaldehyde and 2% glutaraldehyde, 1:1 (v/v), in Tris HCl, pH 7.5, at 4°C.

Anaesthetised chicks (usually 14 days post hatch) were perfused by intracardial injection into the ventricle followed by cannulation of the ascending aortae. During perfusion the descending aorta was clamped. Perfusion pressure was obtained using a gravity feed system with the reservoir containing the fixative maintained at 150 cm. The tubing was primed with ice cold saline before it was attached to the reservoir. After perfusion the brains were excised, placed on microtome chucks and frozen rapidly using liquid nitrogen. The tissue was sectioned as described in Section 9.1.1.1.

9.1.1.3.2 Histochemical GABA-T assay

GABA-T was localised by coupling the oxidation of succinic semialdehyde (SSA), a product of the GABA-T reaction, by succinic semialdehyde dehydrogenase (SSADH) with the reduction of nitroblue tetrazolium (Nitro-BT) to form an insoluble blue formazan (Nitro-$BTH_2$) at the site of the reduction. The reaction which takes place is,

$$\alpha\text{-oxoglutarate} + \text{GABA} \underset{\longleftarrow}{\overset{\text{GABA-T}}{\longrightarrow}} \text{glutamate} + \text{SSA}$$

$$\text{SSA} + NAD^+ + H_2O \underset{\longleftarrow}{\overset{\text{SSADH}}{\longrightarrow}} \text{succinate} + NADH + H^+$$

$$NADH + H^+ + PMS_{(oxid)} \longrightarrow PMS_{(red)} + NAD^+$$

$$PMS_{(red)} + \text{Nitro-BT} \longrightarrow \text{Nitro-}BTH_2 + PMS_{(oxid)}$$

(PMS = phenazine methosulphate).

The sections were incubated in the following medium for 30 minutes at 37°C; Tris maleate (0.1M), pH 7.6 containing 50 mM GABA, 25 mM 2-oxoglutarate (disodium salt), 1.5 mM $NAD^+$, 1.2 mM Nitro-BT, 0.16 mM PMS, 20 mM sodium malonate, 1 mM sodium cyanide, 124 mM sodium chloride and 5 mM magnesium chloride.

Malonate is present in the medium to prevent succinate dehydrogenase activity and sodium cyanide as a respiratory inhibitor. Control sections were incubated in medium containing AOAA (5 mg/ml), a GABA-T inhibitor (Wallach 1961). After incubation the slides were washed briefly in ice cold double distilled water, dehydrated in alcohol, cleared in xylene and mounted in DEPEX as described in 9.1.1.2. Sections were examined and photographed as noted in 9.1.1.2.

Rubenstein and Roberts (1967) demonstrated that the formazan precipitation is specific and, except at very high enzyme activity, proportional to GABA-T activity in brain sections.

Alternate sections were stained with cresyl violet as described in Section 9.1.1.2 for examination of the tissue morphology and identification of the regions containing GABA-T activity.

9.1.2 Biochemical experiments

Chicks were taken at intervals from the first to the fourteenth day post hatch and killed by decapitation. Their brains were rapidly removed and dissected immediately, on ice, into the following regions; hyperstriatum (HS), ectostriatum (ES), archistriatum (AS), midbrain (M) and optic lobes which were divided into the outer tectum (OT) and inner tectal nuclei (OTN).

The cerebellum and optic lobes were separated from the brain. The optic lobes were divided, in the transverse plane, using a razor blade moistened with 0.32M sucrose and the tecta were separated from the central white matter (see Figure 9.1). The telencephalon (forebrain) was separatedfrom the diencephalon (midbrain). The telencephalon was divided into four equal transverse slices using a razor blade moistened with 0.32M sucrose then further dissected, using a fine scalpel blade, into hyperstriatum, ectostriatum and archistriatum (see Figure 9.2). The tissue samples were weighed and each homogenised 1:10 (w/v) in ice cold 0.32M sucrose.

Samples were taken from each of the homogenates and diluted 1:10 (v/v) with ice cold Krebs-Ringer buffer (see Section 3.6.5) to give a final homogenate concentration of 1:100 (w/v). These homogenates were immediately assayed for high affinity uptake of [$^3$H] GABA as described in Section 3.6.5.

Half of each of the remaining homogenates, 1:10 (w/v) in sucrose were kept on ice and the other halves frozen to -20$^{o}$C whilst the uptake assays were being carried out. The homogenates which had been kept on ice, for 45 minutes maximum, were assayed for GAD activity as described in Section 4.1.2. The frozen homogenates were then rapidly thawed and assayed for GABA-T activity as described in Section 3.6.4. Protein concentration was determined by the method of Lowry et al (1951).

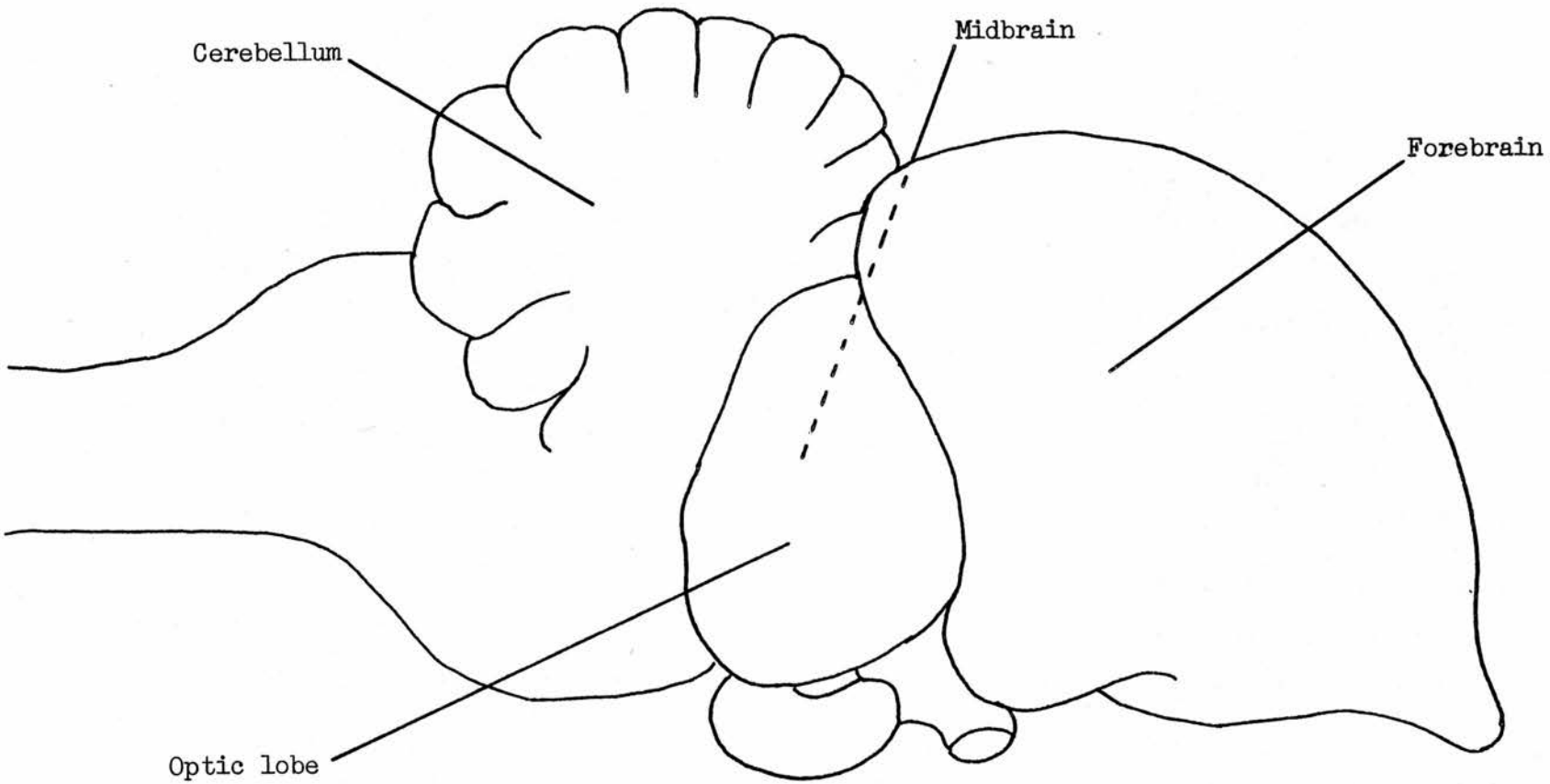


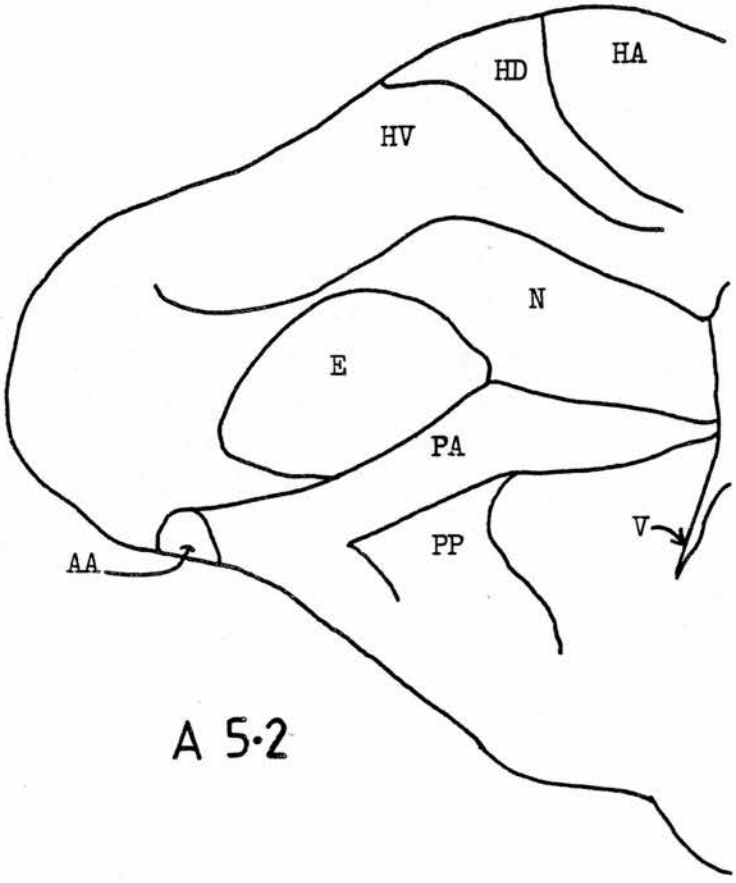

Figure 9.1 Schematic diagram of a lateral view of a chick brain

Figure 9.2. Schematic diagram of a transverse section through chick brain to show the relative positions of the forebrain regions analysed.
(See text, Section 9.1.2 )

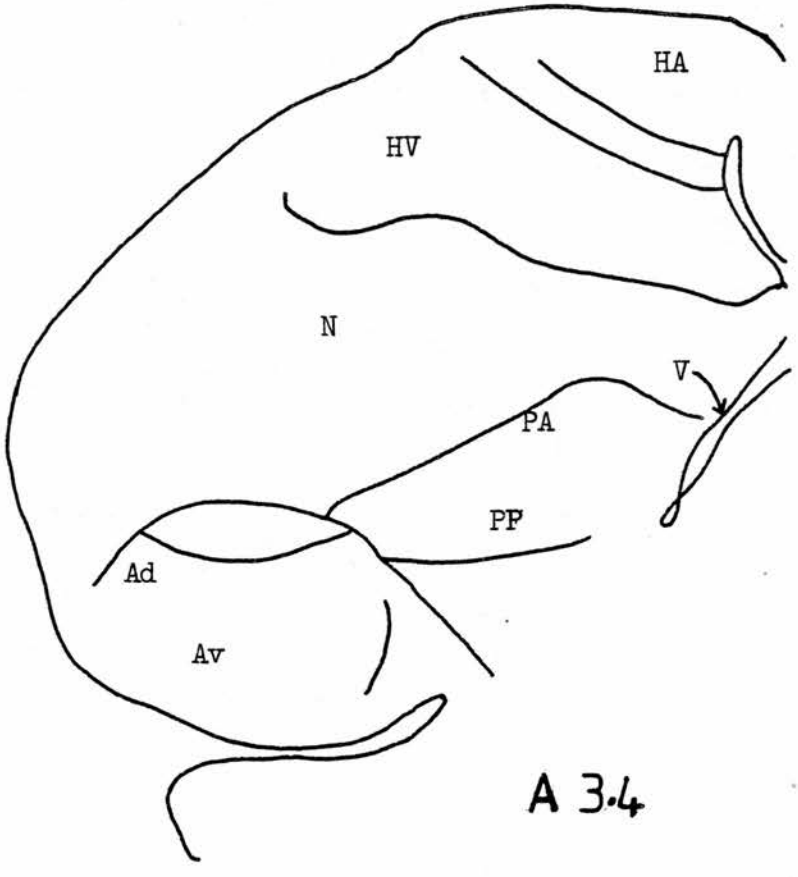
Posterior plane positions according to Youngren and Phillips (1978).

Abbreviations

| | |
|---|---|
| AA | Nucleus archistriatalis anterior |
| Ad | Archistriatum pars dorsalis |
| Av | Archistriatum pars ventralis |
| E | Ectostriatum |
| HA | Hyperstriatum accessorium |
| HD | Hyperstriatum dorsale |
| HV | Hyperstriatum ventrale |
| N | Neostriatum |
| PA | Paleostriatum augmentum |
| PP | Paleostriatum primitivum |
| V | Ventricle |




A 5·2




A 3.4

## 9.2 Results

### 9.2.1 Biochemical

The results obtained when high affinity uptake of [$^3$H] GABA and GAD and GABA-T specific activities were measured in six brain regions, namely OT, OTN, M, HS, AS and ES (see Section 9.1.2) were combined together into two histograms, Figures 9.3 and 9.4.

High affinity uptake of [$^3$H] GABA

High affinity uptake of [$^3$H] GABA was generally greater in the optic lobes and midbrain than in the three forebrain regions examined and declined with age in all regions. This decrease was most noticeable in OT but there was also a marked decrease in OTN and HS. Although GABA uptake remained high in M there was still a gradual decrease over fourteen days. Similarly in AS although high affinity uptake of [$^3$H] GABA decreased with time this decrease was only slight.

GAD specific activity

GAD specific activity was considerably greater in OT, OTN and M than in the forebrain regions examined. In OT and OTN GAD specific activity was approximately 2.5 fold greater. There was a general decrease in specific activity in OT, OTN and M over fourteen days showing a similar trend to that obtained for high affinity uptake of [$^3$H] GABA. However, there was a marked decrease in GAD specific activity on day 3 in these regions. By day 7 specific activity was again at a level slightly lower than that seen on day 1 and continued decreasing with age. In HS and AS GAD specific activity did not change greatly with age remaining at a much lower level than in OT and OTN although the decrease in specific activity at day 3 was still noticeable. In ES, after an initial decrease to day 3, GAD specific activity increased reaching a level, at day 14, similar to that found in the midbrain and optic lobes on that day.

GABA-T specific activity

GABA-T specific activity was slightly higher in optic tectum than in the other regions examined although the difference in activity was not as marked as for GAD. In all regions, except ES, there was a large peak in GABA-T specific activity at day 3. This had decreased by day 7 to a level slightly greater than that found at day 1 and remained constant except in OT where the specific activity increased. In ES there was no very marked fluctuation in GABA-T specific activity, although this was higher on days 3 and 7 than on days 1 and 14.

Figure 9.3. Levels of high affinity uptake of [$^3$H] GABA and GAD and GABA-T specific activities in optic tectum (OT), the tectal nuclei (OTN) and midbrain (M). See Section 9.2.1.

Experimental details are contained in Section 9.1.2. Enzyme specific activity (GAD and GABA-T), 1 unit = 1 nmol product/mg protein/hour. High affinity uptake of [$^3$H] GABA, 1 unit = 1 pmol GABA/mg protein/hour.

GABA-T specific activity (units)
GAD specific activity (units)
High affinity uptake [3H] GABA (units)
0
80
160
240
320
400
0
100
200
300
400
500
0
200
400
500
OT
OTN
M
day 1
day3
day 7
day 14
day 1
day 3
day7
day 14
day1
day3
day7
day14

Figure 9.4. Levels of high affinity uptake of [$^3$H] GABA and GAD and GABA-T specific activities in hyperstriatum (HS), archistriatum (AS) and ectostriatum (ES). See Section 9.2.1.

Experimental details are contained in Section 9.1.2. Enzyme specific activity (GAD and GABA-T), 1 unit = 1 nmol product/mg protein/hour. High affinity uptake of [$^3$H] GABA, 1 unit = 1 pmol GABA/mg protein/hour.

HS
AS
ES
day 1
day 3
day 7
day 14
GABA-T specific activity (units)
GAD specific activity (units)
High affinity uptake [3H]GABA (units)
0
100
200
300
400
500
80
160
240
320

Table 9.1. High affinity uptake of [$^3$H] GABA, and GAD and GABA-T specific activities in the visual system of 14 day old chicks

| | [$^3$H] GABA uptake | GAD | GABA-T |
|---|---|---|---|
| Optic tectum | 175±47 | 136±7 | 274±6 |
| Tectal nuclei | 155±10 | 132±11 | 243±10 |
| Midbrain | 191±19 | 126±15 | 242±26 |
| Hyperstriatum | 146±3 | 77±2 | 176±36 |
| Archistriatum | 188±26 | 61±5 | 239±28 |
| Ectostriatum | 117±14 | 144±6 | 211±25 |

Values are the means ± S.E.M. of 4 determinations. Each determination was carried out in triplicate. Enzyme specific activities are expressed as nmol product/mg protein/hr. High affinity uptake of [$^3$H] GABA is expressed as pmol GABA/mg protein/hr. See Section 9.2.1.

In general high affinity uptake of [$^3$H] GABA paralleled GAD specific activity, i.e. it was higher in those regions, namely OT, OTN and M, where GAD specific activity was higher and tended to decrease with age GABA-T specific activity did not follow this pattern since, with the exception of OT where levels were slightly higher, GABA-T specific activity was similar in all the regions examined. In all these regions GABA-T specific activity was very much higher than GAD specific activity. This difference was particularly noticeable in the two forebrain regions HS and AS.

Table 9.1 lists the values obtained for high affinity uptake of [$^3$H] GABA and GAD and GABA-T specific activities using the brains from 14 day old chicks.

### 9.2.2 Histological

Comparison of the sections stained for GABA-T with the corresponding sections stained with cresyl violet made it possible to identify the regions with high GABA-T activity. The nomenclature used throughout is that of Karten and Hodos (1967). Structures were identified using the chicken brain atlases of van Tienhoven and Juhász (1962) and Youngren and Phillips (1978). Extracts from the atlas of van Tienhoven and Juhász (1962) have been included in Appendix 1. It should be noted that in this study sections were taken in a slightly different plane from that used by van Tienhoven and Juhász (see Appendix 1).

The forebrain was diffusely and fairly evenly stained for GABA-T. It was difficult to distinguish any particular region by the presence of high GABA-T activity, although a number of discrete, deeply stained cells were apparent in paleostriatum just where the lateral forebrain bundle terminates. Comparison between Figures 9.5a and 9.5b shows the extent to which high GABA-T activity is localised in this region.

In midbrain a number of nuclei could be clearly distinguished by high GABA-T activity. These were identified as nucleus rotundus, nucleus ovoidalis (Figures 9.6a and b); nucleus subpraetectalis, nucleus spiriformis lateralis, nucleus isthmo opticus (Figures 9.7a and b); nucleus nervi occulomotorii (Figures 9.8a and b).

Whereas nucleus ovoidalis was uniformly and very deeply stained for GABA-T throughout, nucleus rotundus stained more deeply ventrally. Discrete deeply stained stained cells can be seen throughout both the deeply and less deeply stained areas. Nucleus subpraetectalis is characterised by a cluster of small deeply staining cells whereas the

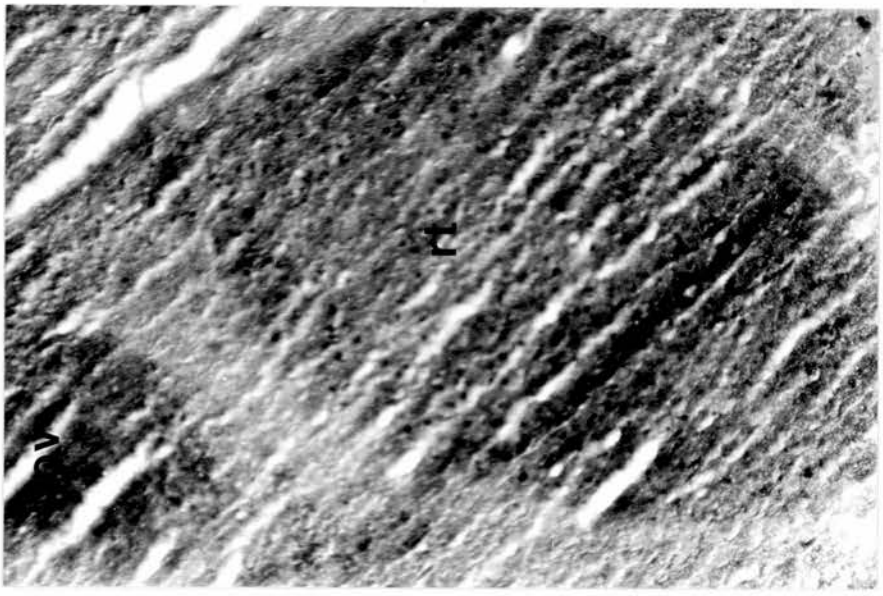
Figure 9.5. Transverse sections through chick forebrain at the level of paleostriatum (pa)

(a) Section stained with cresyl violet for examination of tissue morphology.
(b) GABA-T activity in palestriatum.
See Section 9.1.1 for details of the histological techniques and Appendix 1 for extracts from a chick brain atlas. Scale bar represents 300 μm. Sections were taken consecutively and were 20 μm thick.

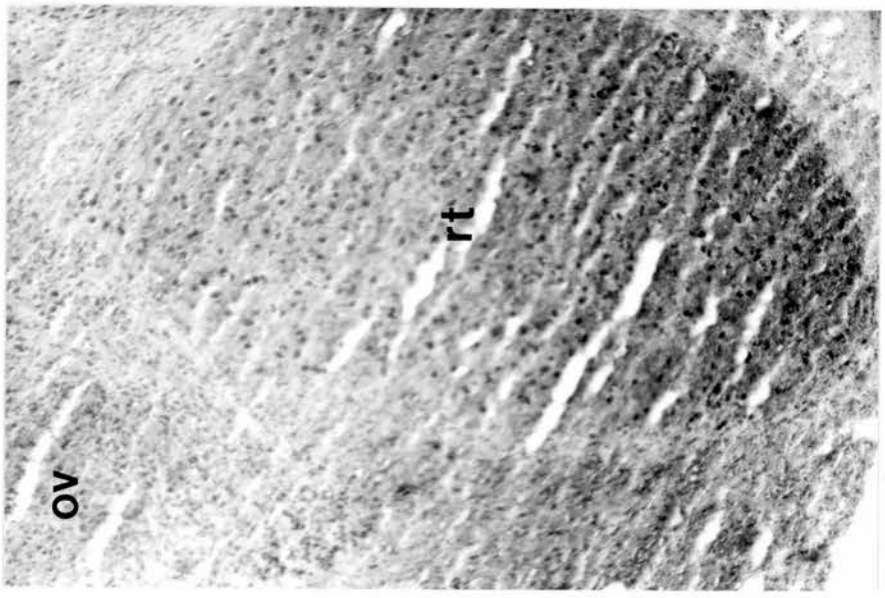
(a) (b)

Figure 9.6. Transverse sections through chick midbrain at the level of nucleus rotundus (rt) and nucleus ovoidalis (ov)

(a) Section stained with cresyl violet for examination of tissue morphology.
(b) GABA-T activity in nucleus rotundus and nucleus ovoidalis.
See Section 9.1.1 for details of histological techniques and Appendix 1 for extracts from a chick brain atlas. Scale bar represents 300 μm. Sections were taken consecutively and were 20 μm thick.




(b)




(a)

Figure 9.7. Transverse sections through chick midbrain at the level of nucleus subpraetectalis (sp), nucleus isthmo opticus (io) and nucleus spiriformis lateralis

(a) Section stained with cresyl violet for examination of tissue morphology.
(b) GABA-T activity in nucleus subpraetectalis, nucleus isthmo opticus and nucleus spiriformis lateralis.
See Section 9.1.1 for details of histological techniques and Appendix 1 for extracts from a chick brain atlas. Scale bar represents 300 μm. Sections were 20 μm thick.

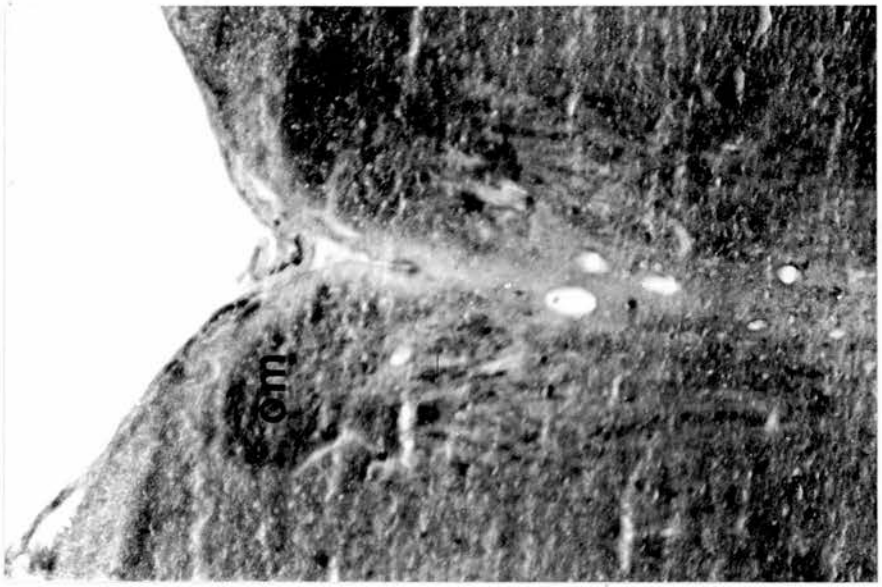
(b)



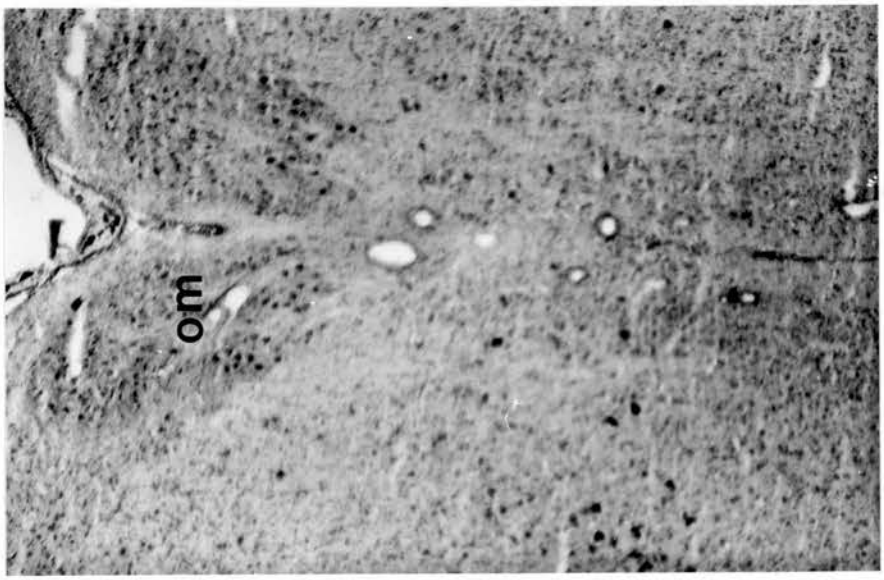

(a)

Figure 9.8. Transverse sections through chick midbrain at the level of nucleus nervi occulomotorii

(a) Section stained with cresyl violet for examination of tissue morphology.
(b) GABA-T activity in nucleus nervi occulomotorii. See Section 9.1.1 for details of histological techniques and Appendix 1 for extracts from a chick brain atlas. Scale bar represents 300 μm. Sections were taken consecutively and were 20 μm thick.




(b)




(a)

Figure 9.9 Transverse sections through chick brain at the level of nucleus isthmi pars magnocellularis (Imc) and Nucleus isthmi pars parvocellularis, (Ipc).

(a) Section stained with cresyl violet for examination of tissue morphology.

(b) GABA-T activity in nucleus isthmi pars magnocellularis and nucleus isthmi pars parvocellularis. See Section 9.1.1. for details of histological techniques and Appendix 1 for extracts from a chick brain atlas. Scale bar represents 300 μm. Sections were taken consecutively and were 20 μm thick.

(a) (b)

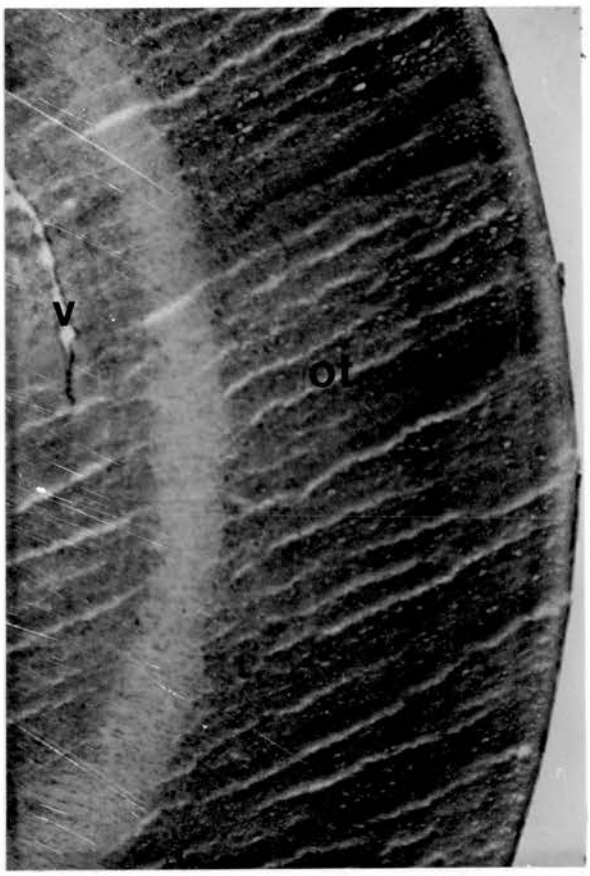



Figure 9.10 Transverse section (20 μm thick) through chick optic lobe. The optic tectum (ot) is deeply stained indicating high GABA-T activity. v= ventricle. Scale bar represents 150 μm.

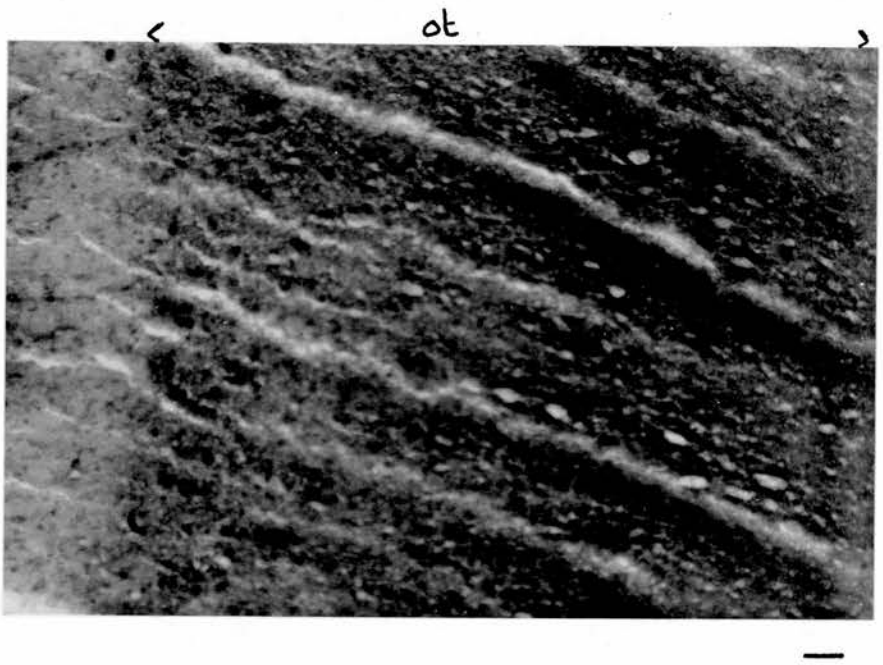



Figure 9.11 Transverse section through chick optic lobe. The optic tectum (6t) is deeply stained throughout indicating high GABA-T activity. In layers I and II, stain is deposited uniformly. In layer III individual cell bodies can be seen. Sections are 20 μm thick. Scale bar represents 60 μm.

part of nucleus spiriformis lateralis which is visible is more diffusely stained. Nucleus isthmo opticus is much less obvious and apparently composed of larger and generally less deeply staining cells.

Nucleus isthmi pars magnocellularis and nucleus isthmi pars parvocellularis also stained very deeply. It is interesting to note that whereas in nucleus isthmi pars parvocellularis the staining is uniform throughout, in nucleus isthmi pars magnocellularis, discrete more deeply staining cells can be seen (Figures 9.9a and b).

In the optic lobe the optic tectum generally stained very deeply indicating very high GABA-T activity, Figure 9.10. In layers I and II the stain was deposited uniformly whereas in deeper layer III individual cell bodies, which were deeply, stained could be seen (Figure 9.11).

## 9.3 Discussion

The aim of this study was to localise GABAergic neurons in chick brain with particular reference to the visual system (see Section 1.13) using two approaches. Firstly to determine biochemically the regional distribution of GABA metabolism by looking at regional variations in high affinity uptake of GABA, a mechanism by which GABA can be removed from the synaptic cleft, (Iversen 1968 & 1971; Iversen & Johnston 1971; Balcar & Johnston 1973; Iversen & Kelly 1975) and of the enzymes of GABA metabolism, GAD which is the enzyme of synthesis and GABA-T the degradative enzyme (see Section 1.6). Secondly it had been intended to use immunocytochemical methods to determine more precisely the localisation of GABAergic neurons using FITC labelled antibody to GAD and thin (20 um) sections of brain. This was to be compared with the distribution of GABA-T activity detected histochemically by the method of Hyde and Robinson (1976).

In studies concerning regional distribution it is not the absolute values for the parameters measured, in this case high affinity uptake of $[^3H]$ GABA and GAD and GABA-T specific activities, which are significant but the relative values when comparisons are made with other regions. This is particularly important in the current study since it has been shown that there is considerable variation in the specific activities of a number of neurotransmitter enzymes, including GAD, when chicks or embryos from different hatches are used (Haywood *et al* 1975; Haywood 1978). Suggested reasons included possible variations in incubation conditions and different parental hens.

The regional distribution of GAD specific activity (Table 9.1) obtained using brains from 14 day old chicks was very similar to that obtained by Vischer *et al* (1982) using brains from adult pigeons. By 14 days post hatch it was considered that any variations due to development would be minimal or absent. Sisken *et al* (1961) showed that GAD and GABA-T specific activity and the concentration of GABA had reached a constant value by day 10 post hatch.

Vischer *et al* (1982) found GAD specific activity to be highest in paleostriatum, a region of the brain not involved in the visual system. Of those regions involved in the visual process which were studied GAD specific activity was reported to be highest and similar in optic tectum,

nucleus rotundus and dorsolateral thalamus, the latter two being midbrain regions. In the Wulst (hyperstriatal region) and ectostriatum GAD specific activity was much lower, less than 50% of the values found in the other regions.

In this study GAD specific activity in optic tectum, the inner tectal nuclei and midbrain were similar and approximately twice the value found in hyperstriatum (Wulst) or archistriatum (see Table 9.1 and Figures 9.3 and 9.4). The results obtained for ectostriatum showed GAD specific activity to be similar to that in optic tectum whereas Vischer et al (1982) found the level more similar to that in hyperstriatum. A possible explanation for this anomaly may be found in the close proximity of ectostriatum and paleostriatum (see Figure 9.2). Contamination of the ectostriatal tissue with a small amount of tissue from paleostriatum, shown to contain high GAD specific activity (Vischer et al 1982) during dissection cannot be totally dismissed.

Haywood (1978) showed that during the last six days of incubation GAD specific activity in five brain regions (optic lobes, anterior and posterior forebrain roofs, forebrain base and thalamus) gradually increased. The most significant increase was during the last day of incubation and first day post hatch when enzyme activity reached a maximum value. In an earlier study (Sisken et al 1961) GAD and GABA-T specific activities and concentration of GABA in optic lobes were studied from day 7 of incubation to day 17 post hatch. Each parameter was shown to increase with time, the maximum value for GAD specific activity being found several days post hatch. At hatching GAD specific activity was half the maximum value. These results contrast with those shown in Figures 9.3 and 9.4 which show that GAD specific activity is highest on day 1 and tends to decrease post hatch, particularly in optic lobes. The exception to this was ectostriatum where by day 14 GAD specific activity was very slightly higher than on day 1, though by only 9%.

Different assay systems were used in the two studies which may be a contributary factor in the conflicting results. The assay used in this study relied on the measurement of $[^{14}C]$ GABA produced from uniformly labelled $[^{14}C]$ glutamate compared with measurement of radioactive carbon dioxide released, again using uniformly labelled $[^{14}C]$ glutamate. Electrophoresis of the reaction products (Section 4.1.3) showed that GABA was the only remaining radiolabelled product.

No such check was carried out in the study of Sisken et al (1961) therefore the production of carbon dioxide, if only in trace amounts, from other metabolic processes cannot be ruled out. Wilson et al (1972) found that the ratio of [$^{14}$C] GABA:$^{14}CO_2$ produced from uniformly labelled GABA was rarely 1:1, with the production of GABA, on occasions, 10% of the carbon dioxide production.

A problem with using high affinity uptake in neurotransmitter studies is the location of the active uptake process since glial cells can also accumulate GABA by a sodium and energy dependent high affinity mechanism (Henn & Hamberger 1971; Schou & Kelly 1974; Shon & Iversen 1974; Iversen and Kelly 1975). Glial cells should account for little of the [$^{3}$H] GABA accumulation in the uptake assay since homogenisation disrupts glial cells. Although the formation of "gliasomes" during homogenisation with the capacity to accumulate GABA has been postulated, the rate of uptake would probably be similar to that of intact glial cells and therefore relatively slow (Schon & Kelly 1974). By using a short incubation such as five minutes, in effect only neuronal uptake would be measured (Schon & Kelly 1975). Experiments carried out in this laboratory involving subcellular fractionation and also L-2,4-diaminobutyric acid, an inhibitor of synaptosomal uptake of GABA, (Schon & Kelly 1974; Iversen & Kelly 1975) suggested that the uptake measured by this method was indeed predominantly synaptosomal and any accumulation by glial elements minimal (S. Wilson, personal communication).

High affinity uptake of [$^{3}$H] GABA declined with age in all the regions examined. This correlates with the findings of Bondy and Purdy (1977) who noted that although high affinity uptake of GABA in chick brain increased during development the peak in activity was at hatching and subsequently activity declined to a level slightly higher than that found just before hatching.

There are similarities in the pattern of distribution of high affinity uptake of GABA and high GAD specific activity. GAD specific activity is higher in those regions where GABA uptake is greater and the changes during development generally more pronounced, namely optic lobe tissue and midbrain. The value for high affinity uptake of GABA recorded for AS on day 14 (Table 9.1) would therefore appear anomalous. However, although at a high level, and comparable with OT, OTN and M for day 14 values, there was little change in high affinity uptake of GABA during development (Figure 9.4). A high value for ES was obtained on day 14.

In contrast to the similar patterns of distribution of GAD and GABA uptake there was no such association found between GABA uptake and GABA-T specific activity which was high in all the regions examined. It was expected that the distribution of high GABA-T specific activity would have some similarity to that of high affinity uptake of GABA since reuptake by inhibitory neurons (Iversen 1968 & 1971; Iversen & Johnston 1971; Balcar & Johnston 1973; Iversen & Kelly 1975) in conjunction with uptake by glial cells (Iversen & Kelly 1975; Schousboe et al 1977) seems the most likely mechanism for removing GABA from the synaptic cleft. In addition high GABA-T specific activity has been shown histochemically to be present in both neurons and glia (Robinson & Wells 1973). Any regional variation in GABA-T activity could not clearly be demonstrated biochemically although the levels of activity were perhaps slightly lower in HS.

Regional variations in GABA-T activity were demonstrated more successfully using histochemical methods. It would appear that there is a general "background" level of GABA-T activity throughout chick brain, although less intense in the forebrain, and that the specific activity is markedly higher in certain specific cells or structures. This is consistent with the observation that glial cells contain low but measureable levels of GABA-T activity accompanied by the ability to accumulate GABA even in tissue that lacks GABA inhibitory neurons (Schon et al 1975). This could explain why no marked regional variation in GABA-T activity could be detected biochemically. GABA-T activity was consistently slightly higher in OT, OTN and M than in the forebrain regions. Since OT stained deeply for GABA-T (Figures 9.10 and 9.11) and both OTN and midbrain contained deeply staining nuclei (Figures 9.6 to 9.9 inclusive) this would be expected.

The fact that GAD specific activity and high affinity uptake of GABA is higher in OT, OTN and M than in the forebrain regions suggests that there is a greater density of GABAergic synapses in those regions. There is evidence suggesting the importance of GABA as a neurotransmitter in avian optic tectum (see 1.13) and the results from this study are in agreement.

It is interesting that GABA-T staining is not uniform throughout optic tectum. In layers I and II which contain intrinsic GABAergic neurons (interneurons) as well as the terminals of GABAergic neurons

which form an inhibitory input from nucleus isthmi pars parvocellularis (Hunt & Kunzle 1976(b); Hunt et al 1977) the staining is more diffuse than in layer III where discrete cell bodies can be seen (Figure 9.11). The deep staining in layers I and II may reflect the greater number of cell bodies and synaptic connections present in those layers whereas in layer III which does not contain such a network of neurons, only discrete cell bodies are stained.

The midbrain contains a number of structures involved in the transmission of visual information including nucleus rotundus and dorsolateral thalamus (Figures 9.6 and 9.7). Although the biochemical evidence pointing to a high level of GABAergic activity in chick midbrain cannot give any indication as to whether this is involved with the visual system, the histochemical GABA-T analysis produced very localised results. Nucleus rotundus was very easily distinguishable (Figure 9.6). Staining was not uniform throughout, the ventral area of the nucleus being much more deeply stained. Individual cell bodies could also be distinguished throughout. This indicates that GABA may have an important role at least in the ventral part of nucleus rotundus.

A detailed analysis of the tectofugal pathway (Benowitz & Karten 1976) suggested that it might consist of five "channels" with cells at various depths within the optic tectum projecting upon distinct subdivisions of nucleus rotundus, in particular a ventral subdivision of nucleus rotundus was found to receive its primary input from two pretectal nuclei; one of which, nucleus subpraetectalis, receives direct retinal input (Karten & Revzin 1966). An indirect inhibitory pathway from the tectum via nucleus subpraetectalis to the ventral subdivision of nucleus rotundus was proposed (Benowitz & Karten 1976) on the basis of their results and those of Granda and Yazulla (1972) who demonstrated that cells in ventral nucleus rotundus showed an inhibitory response to light. Both ventral nucleus rotundus and nucleus subpraetectalis stain deeply for GABA-T, it is possible therefore that GABA may be an inhibitory neurotransmitter involved in this particular inhibitory pathway.

Nucleus subpraetectalis can clearly be seen as a cluster of discrete, deeply stained small cells and nucleus spiriformis lateralis as a cluster of more diffusely stained larger cells. Nucleus isthmo opticus

is just visible at this level as a small cluster of very diffusely stained cells (Figure 9.7). The tectal input from nucleus spiriformis lateralis has been tentatively identified as GABAergic and GABAergic projections have been identified between optic tectum and the pretectum (Hunt & Kunzle 1976(b)). Nucleus isthmo opticus receives an input from the tectum and then sends efferent fibres to the retina.

A number of other nuclei in midbrain, for example nucleus ovoidalis and nucleus nervi acculomotorii (Figures 9.6 and 9.8), obviously contained high levels of GABA-T activity. Since these structures are not involved in the visual pathways any implications which might be drawn from this will not be discussed.

The dissection used in this study resulted in nucleus isthmi pars magnocellularis and nucleus isthmi pars parvocellularis (Figure 9.9) being in the region designated OTN. The high GAD specific activity and GABA uptake found for this region were complemented by very deep staining for GABA-T, particularly in nucleus isthmi pars parvocellularis. In nucleus isthmi pars magnocellularis discrete, very deeply staining cells bodies could also be distinguished whereas in nucleus isthmi pars parvocellularis staining throughout was so pronounced that individual cell bodies could not be seen. A GABAergic input to the optic tectum from nucleus isthmi pars parvocellularis has been postulated (Hunt & Kunzle 1976(b)) and also a GABAergic connection between the rostral and caudal regions of nucleus isthmi para parvocellularis (Hunt et al 1977). The high level of GABA-T activity in this nucleus is demonstrated by the intensity of staining may reflect high level of GABAergic activity.

In the forebrain it was not possible to distinguish any particular region by the histochemical GABA-T stain. The biochemical data suggested that GABAergic activity in the forebrain regions examined was not as great as in the midbrain or optic lobes. From this study it is not possible to speculate as to the extent of the involvement of GABAergic neurons in the visual pathways in chick forebrain.

The biochemical and histochemical data obtained for midbrain and optic lobe correlated with proposed GABAergic pathways in chick visual system after retrograde transport of [$^3$H] GABA (Hunt & Kunzle 1976(b); Hunt et al 1977). High GABA-T activity has been shown to be specifically localised in regions postulated to receive a GABAergic input, for example nucleus subpraetectalis and optic tectum suggesting that, at least at the microscopic level, analysis of distribution of high GABA-T activity could be a useful tool in the preliminary identification of regions containing GABAergic synapses.

More precise biochemical information as to regional distribution of GABA metabolism in chick brain requires dissection and analysis of the individual nuclei. Definitive evidence as to the extent to which GABAergic neurons are involved in the avian visual system might be obtained by immunocytochemistry using specific (FITC labelled) antibodies to chick brain GAD.

APPENDIX 1

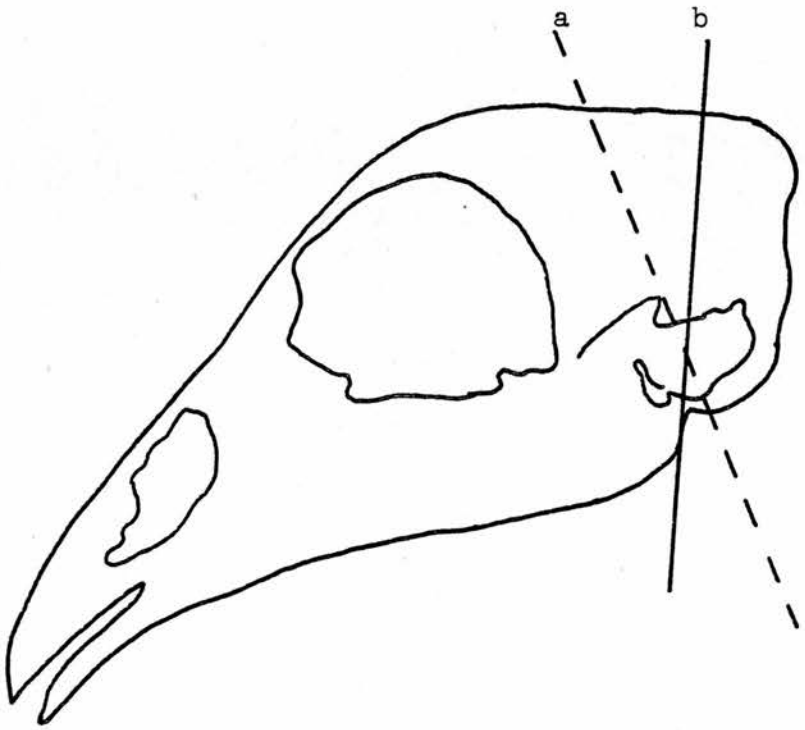



Diagram of the skull of a chick in side view. a, shows the transverse plane in which sections were taken in this study ; b, shows the transverse plane in which sections were taken by van Tienhoven and Juhász (1962).

Extracts from a stereotaxic atlas of chicken brain, van Tienhoven and Juhasz 1962.

*Abbreviations*

(Nomenclature of Huber and Crosby ('29) used unless indicated otherwise.)

AC, Anterior commissure
AE, Area entorhinalis
AN, nu. arcuatus
AP, Area paraentorhinalis
AQ, Aquaduct
ARCH, Archistriatum
AS, Area septalis < lateral nucleus / medial nucleus
AV, Area ventralis (Kuhlenbeck, '38) = tuberculum olfactorium (Jones and Levi-Montalcini)
B, Bed nucleus brachium conjunctivum (cerebellar decussation)
BC, Brachium conjunctivum (Papez) = Superior Cerebellar Decussation (Jungherr)
BP, Brachium pontis (Papez)
BPC, Bed nucleus of pallial commissure
BS, nu. basalis
BT, Bulbo thalamicus (Black)
C, Cortical area or corticoid area
CA, Central acoustic tract (Papez)
CER, Cerebellum
CHI, Optic chiasma
CIO, Capsula interna occipitalis (Schroeder) = (tractus thalamo-frontalis pars caudalis)
CP, Choroid plexus
DA, tr. dorso-archistriaticus (Schroeder)
DI, Decuss. tr. infindibularis
DL, nu. dorsolateralis
DM, nu. dorsomedialis
DMP, nu. dorsomedialis posterior
DMS, nu. dorsolateralis superficialis (Kuhlenbeck, '37) = nu. superficialis parvo cellularis (Jungherr)
DSO, Decussatio supraoptica dorsalis and ventralis
DV, nu. decussationis supraopticae ventralis
E, nu. magnocellularis strati grisei (Edinger, Wallenberg and Holmes)
EB, Eminentia basalis (Haefelfinger) = nu. accumbens + primordial hippocampus (Huber and Crosby) = nu. accumbens + nu. basimedialis (Kuhlenbeck)
EK, Ektostriatum
EM, nu. ectomammilaris
ES, nu. entopeduncularis ventralis superior (Huber and Crosby) = nu. reticularis ventralis of Kuhlenbeck ('37).
FA, tr. fronto-archistriaticus
FLM, Fasciculus longitudinalis medialis
GD, Corpus geniculatus laterodorsalis (Kuhlenbeck, '37)
GLOML, Glomerular layer
GV, Corpus geniculatus lateralis ventralis
HA, Hyperstriatum accessorium
HAM, nu. hypothalamicus anterior medialis
HD, Hyperstriatum dorsale
HI, nu. intercalatus hyperstriaticus
HIP, Hippocampus
HL, nu. habenularis lateralis
HM, nu. habenularis medialis
HP, tr. habenularis peduncularis (Schifferli)
HPM, nu. hypothalamicus posterior medialis
HYP, Hyperstriatum
HYPV, Hyperstriatum ventrale
I, Intercalated part of the posterior commissure
IC, nu. intercalatus (thalami)
IF, tr. infundibularis
IGRL, Inner granular layer
IH, nu. hypothalmic inferior (Kuhlenbeck, '37; Wingstrand)
IM, nu. isthmi pars principalis magnocellularis
IO, tr. isthmo-opticus = tr. tecto isthmicus (Groebbels; Schifferli)
IP, nu. isthmi pars principalis parvocellularis
IS, nu. internus superior (Rendahl) = nu. paraventricularis dorsalis (Kuhlenbeck)
LA, nu. lateralis anterior
LD, nu. lemniscus dorsalis
LFB, Lateral forebrain bundle
LFS, Lamina frontalis superior
LFSm, Lamina frontalis suprema
LH, Lamina hyperstriaticus = tr. fronto occipitalis
LHT, nu. hypothalamicus lateralis (Kuhlenbeck, '37; Wingstrand)
LL, Lemniscus lateralis
LM, nu. subhabenularis medialis
LMD, Lamina medullaris dorsalis
LP, Lateral pontal nucleus
LV, Lateral ventricle
LS, nu. subhabenularis lateralis
M, Medullary part of the posterior commissure
MITL, Mitral layer
ML, nu. mammilaris lateralis
MLD, nu. mesencephalicus lateralis pars dorsalis
MM, nu. mammilaris medialis (Kuhlenbeck, '37; Wingstrand) = nu. mammilaris medialis pars dorsalis (Huber and Crosby)
MP, nu. mesencephalicus profundus
MPL, nu. mesencephalicus profundus pars lateralis
MPt, nu. pontis medialis
MPV, nu. mesencephalicus profundus pars ventralis (Jungherr)
MT, Mesencephalic root of V
nA, nu. annularis of FLM
NB, nu. diagonal band of Broca
nC, nu. c (Rendahl) = nu. intramedialis (Huber and Crosby)
NEOST, Neostriatum
NEOSTF, Neostriatum frontale
nI, nu. interpeduncularis
nIO, nu. isthmo-opticus
nL, nu. linea caudalis
NOC, Nervus oculomotorius
nSM, nu. tr. septomesencephalicus
nT, nu. tuberis (Kuhlenbeck, '37; Wingstrand)
OA, nu. oculomotorius accessorius
OD, nu. oculomotorius dorso medialis
OGRL, Outer granular layer
OL, nu. oculomotorius dorsolateralis
OLFV, Olfactory ventricle
OM, tr. occipito-mesencephalicus
OR, Optic radiation
OV, nu. oculomotorius ventralis (Ariëns Kappers) = nu. oculomotorius ventro medialis (Jungherr)

OVD, nu. ovoidalis
P, Pretectal part of the posterior commissure
PAL, Paleostriatum; p — primitivum, a — augmentatum
PC, Posterior commissure
PD, nu. praeopticus dorsolateralis (Kuhlenbeck, '37) = partly area ventralis
PG, Periventricular gray
PI, nu. paramedianus intermedius (Kuhlenbeck '37) = nu. internus inferior posterior (Huber and Crosby) = nu. internus inferior (Rendahl)
PIT, Pituitary
PLC, Pallial commissure
PO, nu. parageniculatus tecti optici (Kuhlenbeck, '37)
POM, nu. praeopticus medialis
PP, nu. principalis praecommisuralis (Kuhlenbeck, '37)
PPM, nu. praeopticus paraventricularis magnocellularis (Kuhlenbeck, '37) (a) dorsalis, (b) medialis, (c) ventralis
PS, tr. nu. praetectalis-subpraetectalis (Schifferli)
Pt, nu. praetectalis
PV, nu. posteroventralis
PVM, nu. paraventricularis magnocellularis
QF, Quinto-frontalis
R, Raphe
RD, nu. reticularis dorsalis (Kuhlenbeck, '37) = nu. decussationis supraopticae dorsalis (Huber and Crosby)
RET, nu. reticularis superior
ROT, nu. rotundus
RS, Rubrospinal tract (Papez)
RU, nu. ruber
SCT, tr. striotegmentalis et striocerebellaris (Schifferli)
SE, Strat. cellulare externum
SG, Stilus corpus geniculatus (Edinger, Wallenberg and Holmes)
SI, Strat. cellulare internus
SL, nu. semilunaris
SM, tr. septomesencephalicus
SO, Striooculomotorius (Papez)
SOE, nu. supraopticus externus (Legait) = nu. interstitialis magnocellularis lateralis (Huber and Crosby)
SOV, nu. supraopticus ventralis (Legait) = nu. supraopticus (Kuhlenbeck, '37)
SP, tr. striopeduncularis (Papez) = tr. occipito-mesencephalicus (Huber and Crosby)
SpL, nu. spiriformis lateralis
SpM, nu. spiriformis medialis
SPR, nu. subpretectalis
SR, nu. subrotundus
ST, tr. spinotectalis
STH, tr. striothalamicus (Schroeder)
STM, tr. strio medullaris (Huber and Crosby)
T, nu. trochlearis
TC, tr. tectocerebellaris (Groebbels)
TD, nu. tegmentalis dorsalis
TE, tr. tectothalamicus nu. ectomammilaris
TEC, Optic tectum
TFM, tr. thalamo-frontalis medialis
TL, nu, tegmentalis laterodorsalis
TM, tr. thalamomammilaris (Schroeder)
Tn, nu. taenia
TO, tr. nu. ovoidalis
TOM, tr. opticus marginalis
TR, nu. triangularis (nu. tr. habenulo-peduncularis)
TRO, Nervus trochlearis
TS, tr. tectospinalis (Papez)
TT, tr. tectothalamicus
V, Ventricle
VAL, Vallecula
VL, nu. lemmiscus lateroventralis
VLT, nu. ventrolateralis (Kuhlenbeck, '37)
VM, tr. vestibulo-mesencephalicus (Papez)

different from that used by Ralph and Fraps, ('59) because the transverse zero plane in our case is 5 mm posterior to their transverse zero plane.

## MATERIALS AND METHODS

Single Comb White Leghorn hens weighing between 2 and 2.5 kg were used. The following measurements were taken and the ranges indicated in parentheses indicate the ranges of these measurements for birds used in the present studies. All birds in which the parietal bones curved so that the level could not be placed flat on the parietal and frontal bones were discarded. Measurements: (1) Bregma to external occipital crest (Chamberlain, '43) measured along the midsagittal plane (1.20 — 1.60 cm).

(2) Midline to lateral crest (Chamberlain, '43) measured from the middle of the line bregma to external occipital crest (1.15 — 1.40 cm).

(3) Distance between the earplugs when fully inserted into the head (1.1 — 1.3 cm).

Brains were perfused through the carotid arteries with 1% saline and subsequently with 10% buffered formalin. The brains were then fixed in 10% formalin. After fixation for 2–3 days the brains were placed in the stereotaxic instrument and cut in slices of about 1 cm thick along the transverse plane. The cutting was done by using the controls of the stereotaxic instrument (Krieg, '46) after a knife was placed in the electrode holder. Three brains to be used for frozen sections were fixed for two days in buffered 10% formalin and then embedded in gelatin. Frozen sections, 50 μ thick, were made. All sections were saved, mounted on mi-

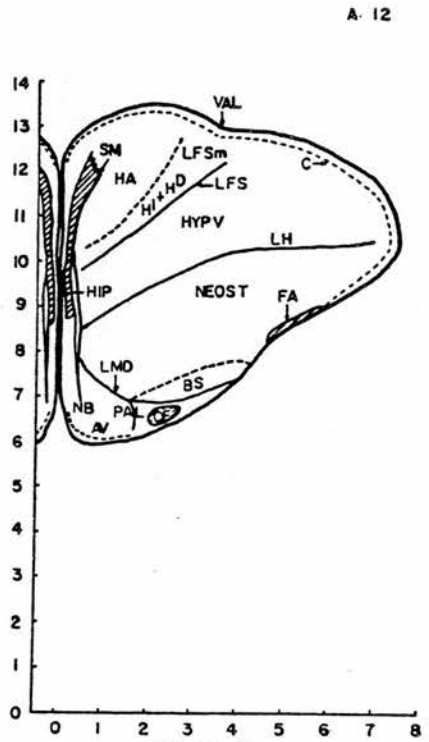



Figure 6

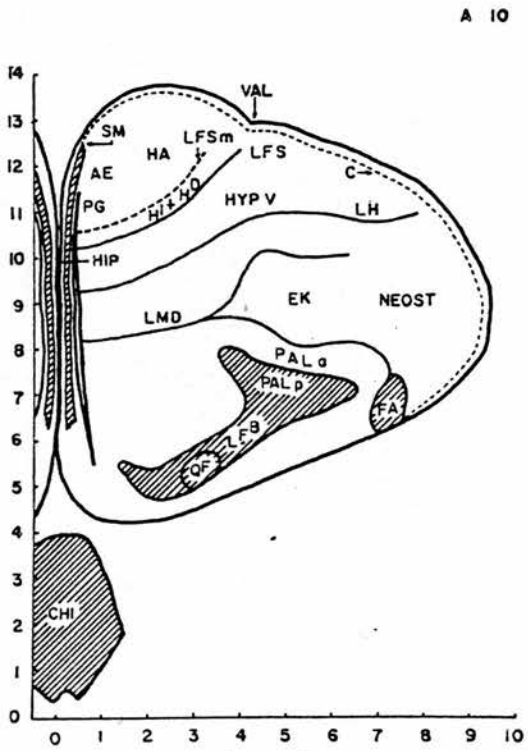



Figure 8

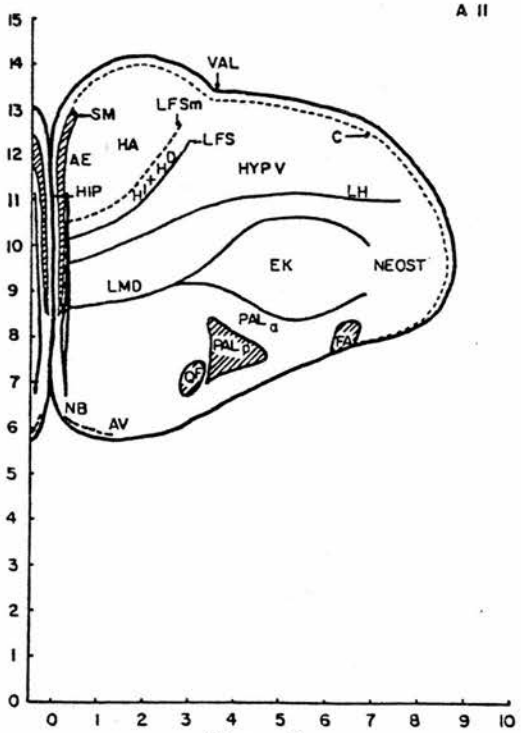



Figure 7

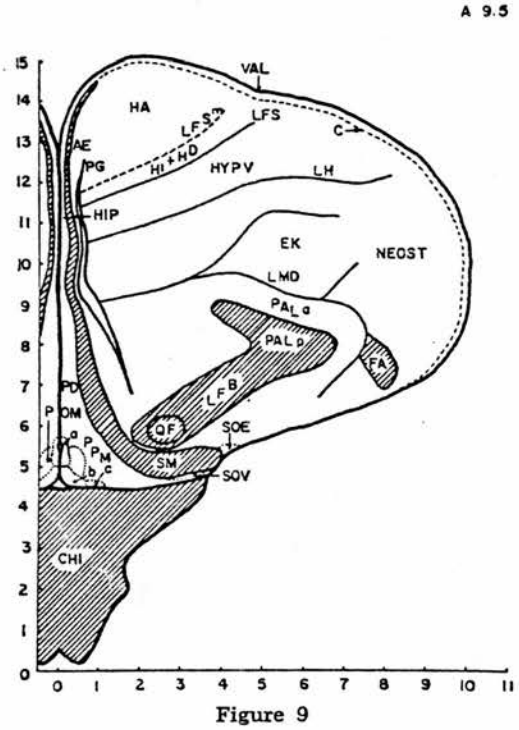



Figure 9

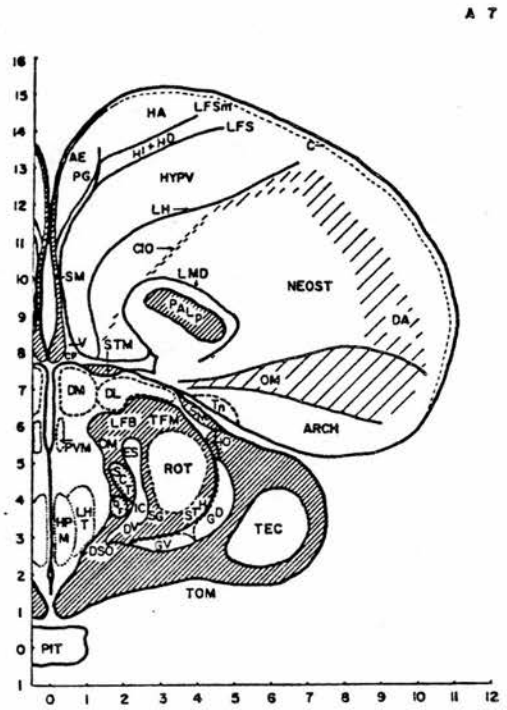



Figure 14

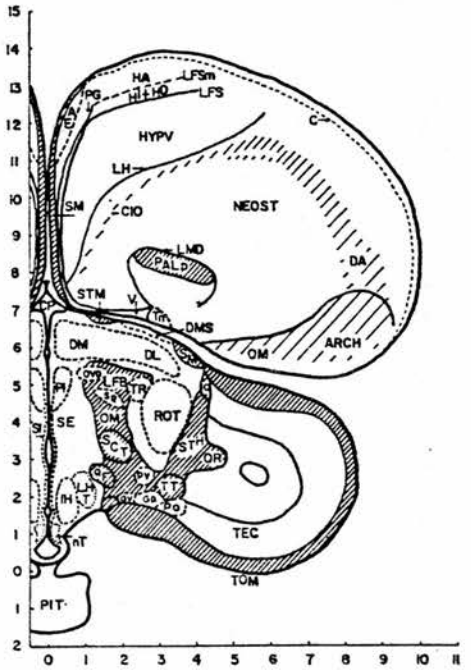



Figure 16

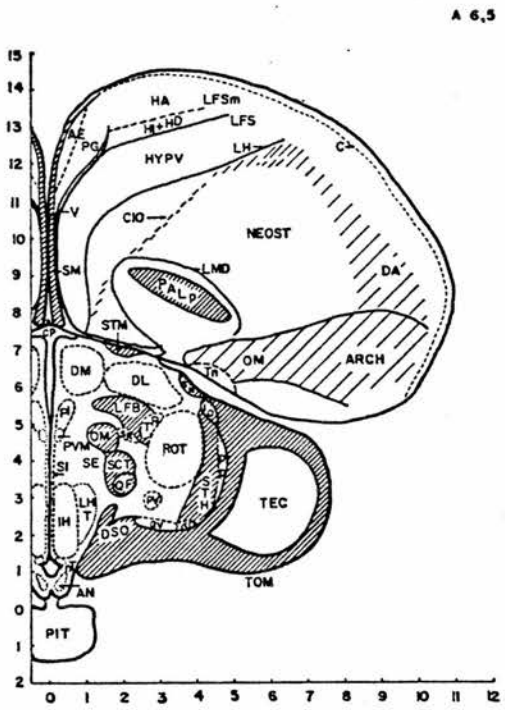



Figure 15

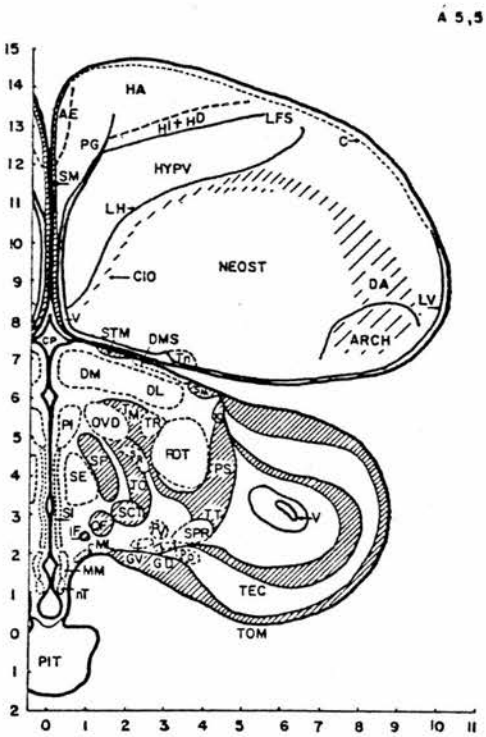



Figure 17

A 3

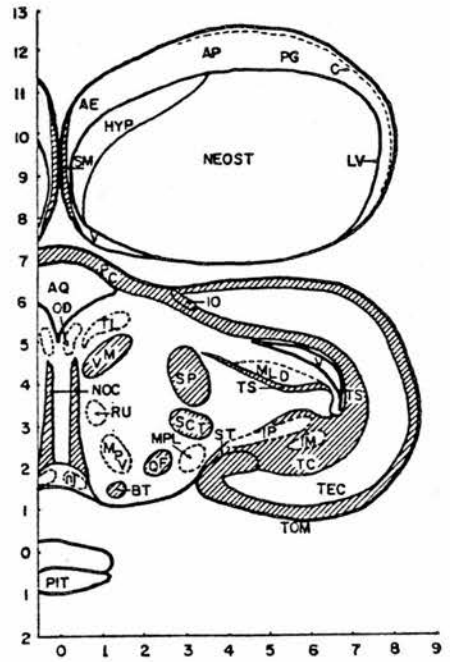



Figure 22

A 1,75

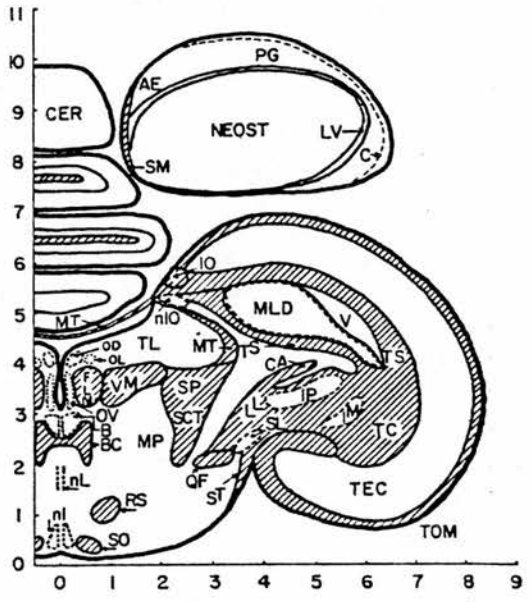



Figure 24

A 2,5

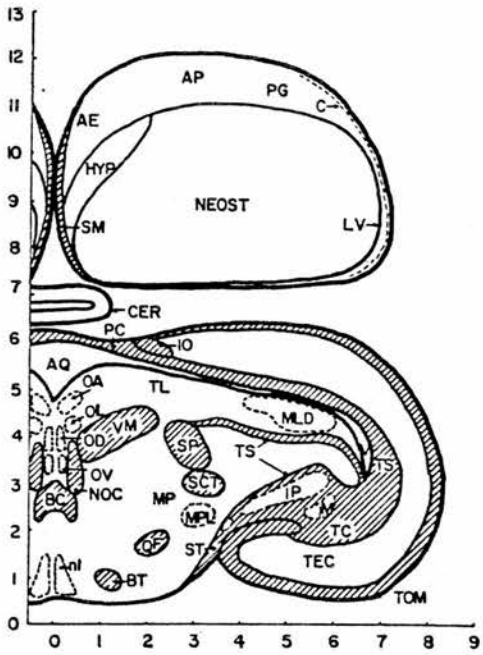



Figure 23

A 1,25

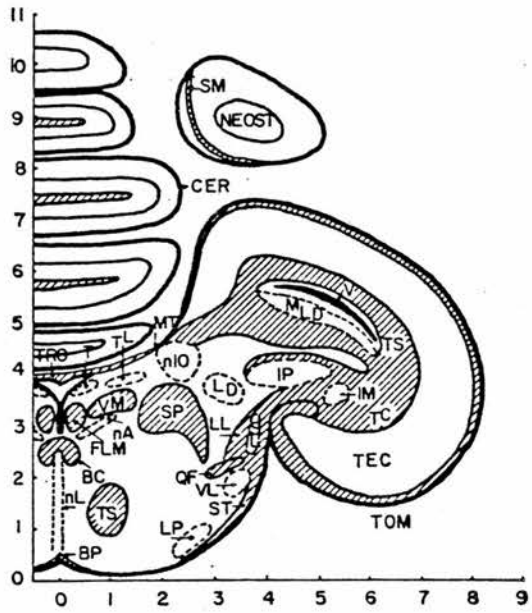



Figure 25

## APPENDIX 2: ABBREVIATIONS

| | |
|---|---|
| AET | 2-aminoethylisothiouronium bromide hydrobromide |
| AOAA | Aminooxy acetic acid |
| AS | Archistriatum |
| bis acrylamide | N,N'-methylene-bis-acrylamide |
| BSA | Serum albumin (bovine) |
| DTT | Dithiothreitol |
| ES | Ectostriatum |
| FITC | Fluorescein isothiocyanate |
| GABA | 4-aminobutyric acid |
| GABA-T | 4-aminobutyrate:2-oxoglutarate transaminase (EC.2.6.1.19) |
| GAD | L-glutamate decarboxylase (EC.4.1.1.15) |
| HAT medium | Selective medium containing hypoxanthine aminopterin and thymidine |
| H(G)PRT | Hypoxanthine(guanine)phosphoribosyl (transferase (EC.2.4.2.8)) |
| HS | Hyperstriatum |
| HT medium | Medium containing hypoxanthine and thymidine |
| LDH | Lactate dehydrogenase |
| M | Midbrain |
| MBTH | 3-methyl-2-benzothiazotrine hydrazone |
| $M_r$ | Relative molecular mass |
| OT | Optic tectum |
| OTN | Optic tectal nuclei |
| PLP | Pyridoxal-5-phosphate |
| PMP | Pyridoxamine phosphate |
| PMP-Sepharose 4B | N'-( -aminohexyl)-pyridoxamine phosphate-Sepharose 4B |
| PMSF | Phenylmethylsulphonyl fluoride |
| PPO | 2,5-diphenyloxazole |
| PRPP | 5-phosphoribosyl-1-pyrophosphate |
| RNAase | Ribonuclease |
| SSA | Succinic semialdehyde |
| SSADH | Succinic semialdehyde dehydrogenase (EC.1.2.1.24) |

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